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(54) Title: TETRACYCLINE DERIVATIVES AND METHODS OF USE THEREOF

(57) Abstract: A treatment for inhibiting microbial or tumor growth is disclosed, which comprises adding to a subject in need of treatment an effective amount of one or more tetracycline derivatives.

TETRACYCLINE DERIVATIVES AND METHODS OF USE THEREOF

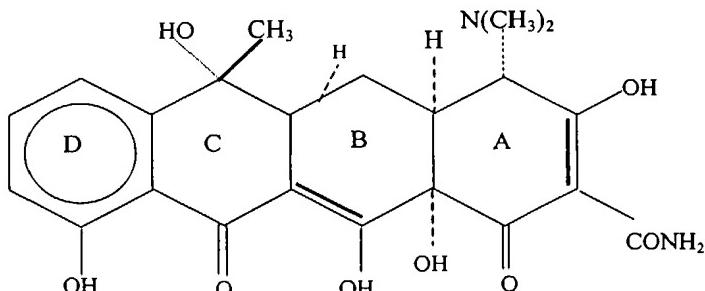
FIELD OF INVENTION

[0001] The present invention relates to novel tetracycline derivatives, methods for producing the novel derivatives and methods of using these derivatives.

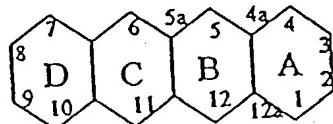
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BACKGROUND OF THE INVENTION

[0002] The compound tetracycline, exhibits the following general structure



[0003] The numbering system of the ring nucleus is as follows:



[0004] Tetracycline as well as the 5-OH (Terramycin) and 7-Cl (Aureomycin) 10 derivatives exist in nature, and are well known antibiotics. Natural tetracycline may be modified without losing their antibiotic properties, although certain elements of the structure must be retained. The modifications that may and may not be made to the basic tetracycline structure have been reviewed by Mitscher in *The Chemistry of Tetracyclines*, Chapter 6, Marcel Dekker, Publishers, New York (1978). According to 15 Mitscher, the substituents at positions 5-9 of the tetracycline ring system may be modified without the complete loss of antibiotic properties. Changes to the basic ring system or replacement of the substituents at positions 1-4 and 10-12, however, generally lead to synthetic tetracyclines with substantially less or effectively no antimicrobial activity. Some examples of chemically modified non-antimicrobial

tetracyclines (hereinafter CMT) are 4-dedimethylaminotetracycline, 4-dedimethylaminosancycline (6-demethyl- 6 -deoxy-4-dedimethylaminotetracycline), 4-dedimethylaminominocycline (7-dimethylamino-4-dedimethylaminotetracycline), and 4-dedimethylaminodoxycycline (5-hydroxy-6-deoxy-4-dedimethyaminotetracycline).

5 [0005] Some 4-dedimethylaminotetracycline derivatives are disclosed in U.S. Pat. Nos. 3,029,284 and 5,122,519. They include 6-demethyl-6-deoxy-4-dedimethylaminotetracycline and 5-hydroxy-6-deoxy-4-dedimethylaminotetracycline with hydrogen and other substituents at C7, and the C9 positions on the D ring. These substitutents include amino, nitro, di (lower alkyl) amino, and mono (lower alkyl) 10 amino or halogen. The 6-demethyl-6-deoxy-4-dedimethylaminotetracycline derivatives and 5-hydroxy-6-deoxy-4-dedimethylaminotetracycline derivatives are said to be useful as antimicrobial agents.

15 [0006] Other 4-dedimethylaminotetracycline derivatives with an oxime group at the C4 position on the A ring are disclosed in U.S. Patent Nos. 3,622,627 and 3,824,285. These oxime derivatives have hydrogen and halogen as substituents at the C7 position and include 7-halo-6-demethyl-6-deoxy-4-dedimethylamino-4-oximinotetracycline, and 7-halo-5-hydroxy-6-deoxy-4-dedimethylamino-4-oximinotetracycline.

20 [0007] Alkylamino (NH-alkyl), and alkylhydrazone (N-NH-alkyl) groups have been substituted on the A ring at the C4 position of 4-dedimethylaminotetracycline. These compounds are known for their antimicrobial properties. See U.S. Patent Nos. 3,345,370, 3,609,188, 3,622,627, 3,502,660, 3,509,184, 3,502, 696, 3,515,731, 3,265,732, 5,122,519, 3,849,493, 3,772,363, and 3,829,453.

25 [0008] In addition to their antimicrobial properties, tetracyclines have been described as having a number of other uses. For example, tetracyclines are also known to inhibit the activity of collagen destructive enzymes, such as matrix metalloproteinases (MMP), including collagenases (MMP-1), gelatinase (MMP-2) and stromelysin (MMP-3). Golub et al., *J. Periodont. Res.* 20:12-23 (1985); Golub et al. *Crit. Revs. Oral Biol. Med.* 2:297-322 (1991); U.S. Pat. Nos. 4,666,897; 4,704,383; 30 4,935,411; 4,935,412. Also, tetracyclines have been known to inhibit wasting and protein degradation in mammalian skeletal muscle, U.S. Pat. No. 5,045,538, and to enhance IL-10 production in mammalian cells.

[0009] Furthermore, tetracyclines were reported to enhance bone protein synthesis in U.S. Pat. No. Re. 34,656, and to reduce bone resorption in organ culture in U.S. Pat. No. 4,704,383.

[00010] Similarly, U.S. Pat. No. 5,532,227 to Golub et al, discloses that 5 tetracyclines can ameliorate the excessive glycosylation of proteins. In particular, tetracyclines inhibit the excessive collagen cross linking which results from excessive glycosylation of collagen in diabetes.

[00011] Tetracyclines are known to inhibit excessive phospholipase A₂ activity involved in inflammatory conditions such as psoriasis as disclosed in U.S. Pat. No. 10 5,532,227. In addition, tetracyclines are also known to inhibit cyclooxygenase-2 (COX-2), tumor necrosis factor (TNF), nitric oxide and IL-1 (interleukin-1).

[00012] These properties cause the tetracyclines to be useful in treating a number of diseases. For example, there have been a number of suggestions that tetracyclines, including non-antimicrobial tetracyclines, are effective in treating arthritis. See, for 15 example, Greenwald, et al. "Tetracyclines Suppress Metalloproteinase Activity in Adjuvant Arthritis and, in Combination with Flurbiprofen, Ameliorate Bone Damage," *Journal of Rheumatology* 19:927-938 (1992); Greenwald et al., "Treatment of Destructive Arthritic Disorders with MMP Inhibitors; Potential Role of Tetracyclines in Inhibition of Matrix Metalloproteinases: Therapeutic Potential," 20 *Annals of the New York Academy of Sciences* 732:181-198 (1994); Kloppenburg, et al. "Minocycline in Active Rheumatoid Arthritis," *Arthritis Rheum* 37:629-636 (1994); Ryan et al., "Potential of Tetracycline to Modify Cartilage Breakdown in Osteoarthritis," *Current Opinion in Rheumatology* 8:238-247 (1996); O'Dell et al, "Treatment of Early Rheumatoid Arthritis with Minocycline or Placebo," *Arthritis 25 Rheum* 40:842-848 (1997).

[00013] Tetracyclines have also been suggested for use in treating skin diseases. For example, White et al., *Lancet*, Apr. 29, p. 966 (1989) report that the tetracycline minocycline is effective in treating dystrophic epidermolysis bullosa, which is a life-threatening skin condition believed to be related to excess collagenase.

30 [00014] Furthermore, studies have also suggested that tetracyclines and inhibitors of metalloproteinases inhibit tumor progression, DeClerck et al., *Annals N.Y. Acad. Sci.*, 732:222-232 (1994), bone resorption, Rifkin et al., *Annals N.Y. Acad. Sci.*,

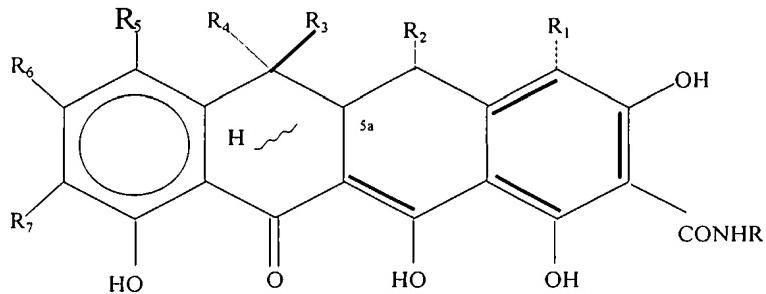
732:165-180 (1994), angiogenesis, Maragoudakis et al., *Br. J. Pharmacol.*, 111:894-902 (1994), and may have anti-inflammatory properties, Ramamurthy et al., *Annals N.Y. Acad. Sci.*, 732, 427-430 (1994).

[00015] Based on the foregoing, tetracyclines have been found to be effective in 5 treating numerous diseases and conditions. Therefore, there is a need for new and even more useful derivatives.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

[00016] Compounds disclosed herein are derivatives of tetracycline, which exhibit 10 antimicrobial and/or anti-cancer activity.

[00017] In a preferred embodiment, the present invention relates to a treatment for inhibiting microbial or tumor growth which comprises adding to a subject in need of said treatment an effective amount of a tetracycline composition or salt thereof, comprising a general formula:



15

wherein R is selected from $\text{CH}_2\text{N}(\text{R}_a\text{R}_b)$ or $\text{CH}_2\text{N} \begin{array}{c} \text{R}_c \\ \diagup \\ \diagdown \\ \text{R}_d \end{array} \text{X}$;

20 R_aR_b are C_1 to C_6 alkyl;

R_c and R_d are $(\text{CH}_2)_n\text{CHR}_e$, n is 0 or 1 and R_e is H, C_1 to C_6 alkyl, or NH_2 , and X is NH, S, or CH_2 ;

R_1 is H or OH;

25 R_2 is H, OH, =O, or OCOR_8 , where R_8 is C_1 to C_6 alkyl, or alternatively, R_2 is $\text{N}(\text{R}_9)_2$, where R_9 is hydrogen or C_1 to C_6 lower alkyl, or R_9CO , with the proviso that when R_2 is keto or $\text{N}(\text{R}_9)_2$, then R_3 and R_4 are H or CH_3 , and R_3 and R_4 are not both CH_3 or H;

5a hydrogen is α or β ;

R₃ and R₄ are H, CH₃ or F, with the proviso that when R₃ is CH₃, then R₄ is H or F, and when R₄ is CH₃, then R₃ is H or F and R₄ and R₅ are not both F;

5 R₅ and R₇ are halogen, H, NO₂, N₃, N₂⁺, C₂H₅OC(S)S-, CN, NR₁₀R₁₁, where R₁₀ and R₁₁ are H, C₁ to C₁₀ alkyl, and R₁₂(CH₂)_nCO-, where n is 0 to 5 and R₉ is H, NH₂, mono or disubstituted amino selected from straight, branched or cyclized C₁ to C₁₀ alkyl groups, with the proviso that R₁₀ and R₁₁ are not both R₁₂(CH₂)_nCO-;

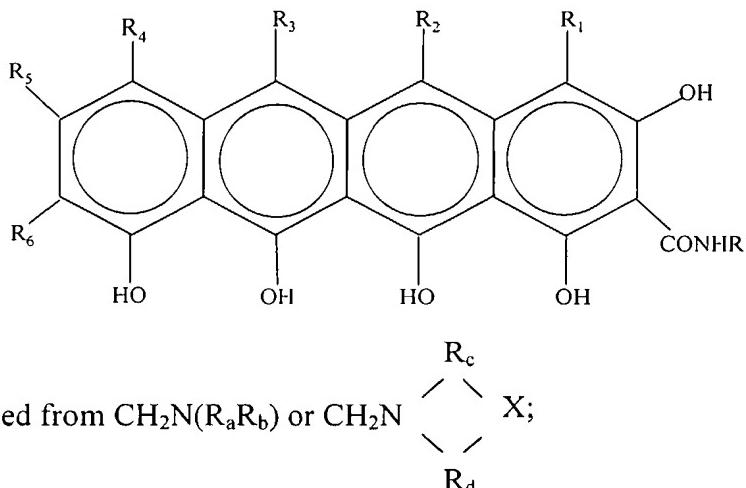
R₇, alternatively, is tertiary butyl; and

R₆ is halogen, acetylene, R₁₃-C₆H₅, where R₁₃ is NO₂, halogen, acetylamino, amino,

10 phenyl, alkyl, or alkoxy.

[00018] In a further preferred embodiment, the present invention relates to a treatment for inhibiting microbial or tumor growth which comprises adding to a subject in need of said treatment an effective amount of a tetracycline composition or salt thereof, comprising a general formula:

15



20 where R_aR_b are C₁ to C₆ alkyl;

R_c and R_d are (CH₂)_nCHR_c, n is 0 or 1 and R_c is H, C₁ to C₆ alkyl, or NH₂, and X is NH, S, or CH₂;

R₁ is H or OH;

R₂ is H or OH;

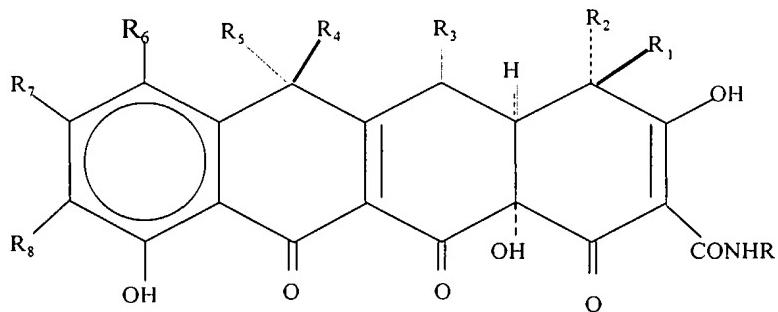
25 R₃ is H or CH₃;

R₄ and R₆ are halogen, H, NO₂, N₃, N₂⁺, C₂H₅OC(S)S-, CN, NR₇R₈, where R₇ and R₈ are H, C₁ to C₁₀ alkyl, and R₉(CH₂)_nCO-, where n is 0 to 5 and R₉ is H, NH₂, mono or

disubstituted amino selected from straight, branched or cyclic C₁-C₁₀ alkyl, with the proviso that R₇ and R₈ are not both R₉(CH₂)_nCO-; and

R₆ is H or halogen, acetylene, R₁₀-C₆H₅, where R₁₀ is NO₂, halogen, acetylarnino, amino, phenyl, alkyl, or alkoxy.

- 5 [00019] In a further preferred embodiment, the present invention relates to a treatment for inhibiting microbial or tumor growth which comprises adding to a subject in need of said treatment an effective amount of a tetracycline composition or salt thereof, comprising a general formula:



10

wherein R is selected from CH₂N(R_aR_b) or CH₂N X;

where R_aR_b are C₁-C₆ alkyl;

15 R_c and R_d are (CH₂)_nCHR_e, n is 0 or 1 and R_e is H, C₁ to C₆ alkyl, or NH₂, and X is NH, S, or CH₂;

R₁ and R₂ are H or N(R₉)₂, where R₉ is H or C₁ to C₆ alkyl with the proviso that R₁ and R₂ are not both N(R₉)₂, R₁ and R₂ are also N(R₁₀)₃I, where R₁₀ is C₁ to C₆ alkyl, with the proviso that R₁ and R₂ are not both N(R₁₀)₃I;

20 R₃ is H;

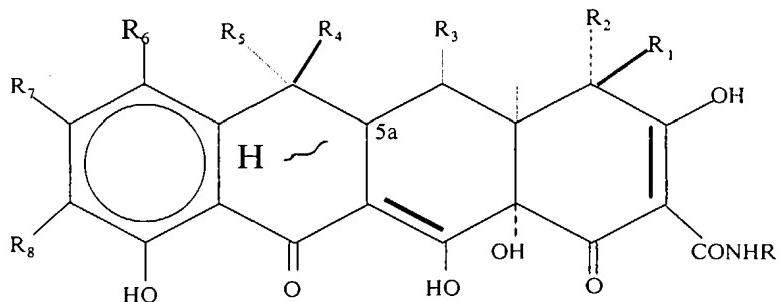
R₄ and R₅ are H, CH₃ or F, with the proviso that when R₄ is CH₃, then R₅ is H or F, and when R₅ is CH₃, then R₄ is H or F, and R₄ and R₅ are not both F;

R₆ and R₈ are halogen, H, NO₂, N₃, N₂⁺, C₂H₅OC(S)S-, CN, NR₁₁R₁₂, where R₁₁ and R₁₂ are H, C₁ to C₁₀ alkyl, and R₁₃(CH₂)_nCO-, where n is 0 to 5 and R₁₃ is H, NH₂,

25 mono or disubstituted amino selected from straight, branched or cyclized C₁-C₁₀ alkyl groups, with the proviso that R₁₀ and R₁₁ are not both R₁₂(CH₂)_nCO-; and

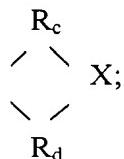
R₈ can also be tertiary butyl and R₇ is H or halogen.

[00020] In an additional preferred embodiment, the present invention relates to a treatment for inhibiting microbial or tumor growth which comprises adding to a subject in need of said treatment an effective amount of a tetracycline composition or salt thereof, comprising a general formula:



5

wherein R is selected from $\text{CH}_2\text{N}(\text{R}_a\text{R}_b)$ or CH_2N



R_a R_b are C_1-C_6 ;

10

R_c and R_d are $(CH_2)_n CHR_e$, n is 0 or 1, R_e is H, alkyl (C_1 to C_6), NH_2 , and X is NH, S, or CH_2 ;

R₁ and R₂ are H or N(R₉)₂ where R₉ is hydrogen or lower alkyl (C₁ to C₆), with the proviso that R₁ and R₂ can not both be N(R₉)₂.

R_3 is H, OH, =O, OCOR₁₀, where R₁₀ is C₁ to C₆, or alternatively, R_3 is N(R₉)₂ where R₉ is hydrogen or C₁ to C₆, with the proviso that when R₂₃ is =O or N(R₉)₂, then R₄ and R₅ are H or CH₃ and R₄ and R₅ are not both CH₃ or H;

20

5a hydrogen is α or β ;

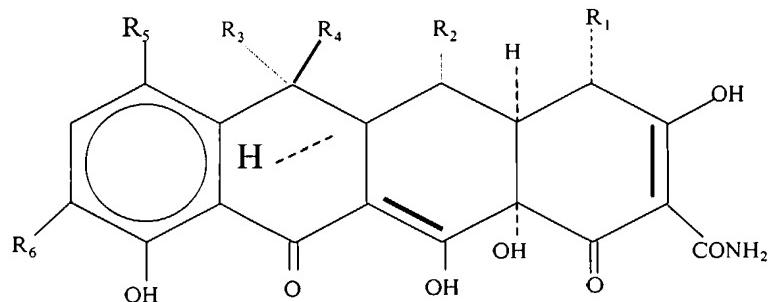
R_4 and R_5 are H, CH_3 or F, with the proviso that when R_4 is CH_3 , then R_5 is H or F, and when R_5 is CH_3 then R_4 is H or F and R_4 and R_5 are not both F;

R₆ and R₈ are halogen, H, NO₂, N₃, N₂⁺, C₂H₅OC(S)S-, CN, NR₁₀R₁₁, where R₁₀ and R₁₁ are H, alkyl (C₁ to C₁₀), and R₁₂ (CH₂)_nCO-, where n is 0 to 5 and R₁₂ is H, NH₂, mono or disubstituted amino selected from straight, branched or cyclized C₁ to C₁₀

groups, with the proviso that R₁₀ and R₁₁ are both R₁₂ (CH₂)_nCO-, or alternatively, R₈ is tertiary butyl;

R₇ is H or halogen, acetylene, R₁₂-C₆H₅, where R₁₂ is NO₂, halogen, acetylarnino, amino, phenyl, alkyl, or alkoxy.

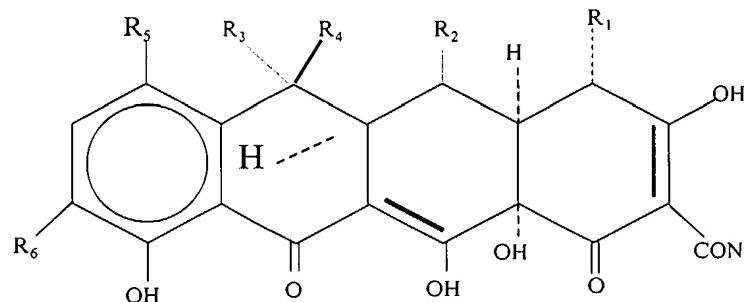
- 5 [00021] In a further preferred embodiment, the present invention relates to a treatment for inhibiting microbial or tumor growth which comprises adding to a subject in need of said treatment an effective amount of a tetracycline composition or salt thereof, comprising a general formula:



10

wherein R₁ is H or N(CH₃)₂; R₂ is H, CH₃ or OCOCH₃; R₃ is H or CH₃; R₄ is H; R₅ is H, N(CH₃)₂, NH₂, N(C₄H₉)₂, N(C₆H₁₃)₂, N(3,3 - dimethylbutyl)₂, N₃, N₂⁺, NO₂, NHCOCH₃, NH(n-propyl)₂, NH-isobutyl, NH-isobutylmethyl, NH(cyclobutyl), NH(cyclobutyl methyl); and R₆ is H, NO₂, NH₂, (CH₃)₂CHNH, N₃, NHCOCH₃, N₂⁺, N₃ or (CH₃)₃C.

- 15 [00022] In a further preferred embodiment, the present invention relates to a treatment for inhibiting microbial or tumor growth which comprises adding to a subject in need of said treatment an effective amount of a tetracycline composition or salt thereof, comprising a general formula:



wherein R₁ is H or N(CH₃)₂; R₂ is H, OH or OCOCH₃; R₃ is H or CH₃; R₄ is H; R₅ is H, N(CH₃)₂; N(C₄H₉)₂, N(C₆H₁₃)₂, N(3,3-dimethylbutyl)₂; N₃, N₂⁺, NO₂, NHCOCH₃, 5 NH(n-propyl)₂, NH-isobutyl, NH-isobutylmethyl, N(CH₃CO)(isobutyl), NH(cyclobutyl), NH(cyclobutyl methyl), or NH₂; and R₆ is H, NO₂, NH₂, (CH₃)₂ CHNH, N₃, NHCOCH₃, N₂⁺, or (CH₃)₃C.

[00023] The following examples describe the preparation of various substances in
10 accordance with the present invention.

[00024] HPLC analyses were performed on Waters reverse phase C₁₈-symmetry columns using water (containing 0.1% TFA) and acetonitrile as eluents

[00025] Analytical LC/MS runs were performed on a Hewlett Packard Series 1100 liquid chromatographer equipped with a series 1100 MSD detector, with ES 15 ionization mode and UV detection at 270 nm, using a 4.6 x 100 mm column with a flow rate of 0.4 mL/min. Preparative HPLC was performed on a Gilson serial HPLC with UV detection at 270 nm, using a 19 x 150 mm column and a flow rate of 15 mL/min. ¹H NMR were recorded on a Bruker 300 MHz spectrometer in deuterated methanol.

20

9-Nitro-doxycycline sulfate

[00026] To a stirred solution of doxycycline hydrochloride (1 g, 2.08 mmol) in 30 mL of concentrated sulfuric acid at 0 °C was added potassium nitrate (252 mg, 2.49 mmol). The resulting mixture was stirred for 20 min at 0 °C, then poured into 1.2 L 25 cold ether with stirring. The solid which precipitated was filtered out, washed with cold ether and dried under vacuum, and 1.22 g of cream colored product was obtained. If needed, the product was purified by dissolving in methanol, filtering and pouring the filtrate into ether. The solid which separated was filtered out and dried.

30 9-Amino-doxycycline sulfate

[00027] To a stirred solution of 9-nitro-doxycycline sulfate (1 g, 1.7 mmol) in 35 mL of ethylene glycol monomethyl ether and 5 mL of methanol was added 700 mg of 10% Pd/C. The reaction mixture was hydrogenated at atmospheric pressure for 6 hrs. The catalyst was filtered off through celite washing with methanol. The filtrate was 5 concentrated, then added dropwise to 800 mL cold ether with stirring. The solid that separated was filtered out, washed with cold ether and dried under vacuum, and 843 mg of cream colored product was obtained.

9-Isopropylamino-doxycycline sulfate

[00028] To a stirred solution of 9-amino-doxycycline sulfate (100 mg, 0.18 mmol) on 10 mL of ethylene glycol monomethyl ether was added 0.05 ml of concentrated sulfuric acid, 0.5 mL acetone and 100 mg of 10% Pd/C. The resulting mixture was hydrogenated at atmospheric pressure for 1 h 15 min, then the catalyst was filtered through celite, washing with methanol. The filtrate was concentrated, then poured into 150 mL of cold ether with stirring. The solid that separated was filtered out, washed with cold ether and dried under vacuum, and 109 mg of cream colored product was obtained. The product was purified by dissolving in methanol, filtering and pouring the filtrate into ether. The solid which formed was filtered out and dried.

9-Acetamido-doxycycline sulfate

[00029] To a stirred solution of 9-amino-doxycycline sulfate (72 mg, 0.13 mmol) in 1.5 mL of 1,3-dimethyl-2-imidazolidinone were added 70 mg sodium bicarbonate followed by 0.05 mL of acetyl chloride. The mixture was stirred at room temperature for 1 hour and then filtered. The filtrate was concentrated and poured into 100 mL cold ether with stirring. The solid that separated was filtered out and dried under vacuum, and 92 mg was obtained.

9-Diazonium-doxycycline sulfate hydrochloride

[00030] To a stirred solution of 9-amino-doxycycline sulfate (300 mg, 0.54 mmol) in 9 mL of 0.1N hydrochloric acid in methanol cooled in an ice bath was added 0.3 mL of n-butyl nitrite. The solution was stirred at 0 °C for 30 minutes then poured into

300 mL of cold ether with stirring. The solid that separated was filtered out and dried under vacuum, and 256 mg of an orange-brown product was obtained.

9-Azido-doxycycline sulfate

5 [00031] To a stirred solution of 9-diazonium-doxycycline sulfate hydrochloride (80 mg, 0.14 mmol) in 4.5 mL of 0.1N hydrochloric acid in methanol was added 10 mg of sodium azide. The solution was stirred at room temperature for 2 hours, then poured into 100 mL of cold ether with stirring. The solid that separated was filtered out and dried under vacuum, and 43 mg of product was obtained.

10

9-(4-fluorophenyl)-doxycycline

[00032] To a solution of 9-diazonium-doxycycline sulfate hydrochloride (358 mg, 0.59 mmol) and 4-fluorophenyl boronic acid (107 mg, 0.76 mmol) in 4 mL of methanol was added 18 mg of palladium(II)acetate and the resulting mixture was stirred at 15 room temperature for 16 hours. The catalyst was filtered off and the residue was concentrated and purified by preparative HPLC. The collected fractions from HPLC were concentrated and poured into 20 ml of cold ether. The solid that was separated was filtered out and dried under vacuum, and 32 mg of product was obtained.

20 7-[1,2-bis(carbobenzyloxy)hydrazino]-doxycycline

[00033] To a stirred solution of doxycycline hydrochloride (300 mg, 0.62 mmol) in 2.5 ml of THF and 3.2 mL of methanesulfonic acid at 0 °C was added 230 mg of dibenzyazodicarboxylate. The resulting mixture was stirred at 0 °C for 2 hours, then poured into 400 mL of cold ether. The solid that separated was filtered out and dried 25 under vacuum, and 479 mg of an off-white product was obtained.

7-Amino-doxycycline

[00034] To a solution of 7-[1,2-bis(carbobenzyloxy)hydrazino]-doxycycline (478 mg) in 30 mL of ethylene glycol monomethyl ether was added 200 mg of 10% Pd/C and the resulting mixture was hydrogenated for 3 hours at room temperature. The catalyst was filtered through celite washing with methanol and the filtrate was

concentrated and poured into 500 mL cold ether. The solid that separated was filtered out and dried under vacuum, and 268 mg of product was obtained.

7-Dimethylamino-doxycycline sulfate

- 5 [00035] To a solution of 7-amino-doxycycline (100 mg) in 8 mL of ethylene glycol monomethyl ether were added 1 mL of a 37% aqueous formaldehyde solution, 0.05 mL of concentrated sulfuric acid and 100 mg of 10% Pd/C. The resulting mixture was hydrogenated at atmospheric pressure for 6 hours, then the catalyst was filtered through celite, washing with methanol. The residue was concentrated and poured into
10 100 ml cold ether with stirring. The solid that separated was filtered out with difficulty, being very gluey. The solid was immediately redissolved in methanol and poured into 100 ml cold ether with stirring. The solid that separated was filtered out and dried under vacuum, and 89 mg of product was obtained.

15 7-Dipropyl-doxycycline sulfate

- [00036] To a solution of 7-amino-doxycycline (90 mg) in 8 mL of ethylene glycol monomethyl ether were added 0.5 mL of propionaldehyde, 0.05 mL of concentrated sulfuric acid and 80 mg of 10% Pd/C. The resulting mixture was hydrogenated at atmospheric pressure for 5 hours, then the catalyst was filtered through celite, washing with methanol. The residue was concentrated and poured into 100 ml cold ether with stirring. The solid that separated was filtered out and dried under vacuum, and 415 mg of product was obtained.

7-Dibutylamino-doxycycline sulfate

- 25 [00037] To a solution of 7-amino-doxycycline (100 mg, 0.2 mmol) in 6 mL of ethylene glycol monomethyl ether were added 0.6 mL of butyraldehyde, 0.05 mL of concentrated sulfuric acid and 100 mg of 10% Pd/C. The resulting mixture was hydrogenated at atmospheric pressure for 4 hours, then the catalyst was filtered through celite, washing with methanol. The residue was concentrated and poured into
30 100 ml cold ether with stirring. The solid that separated was filtered out and dried under vacuum, and 103 mg of product was obtained.

7-Di(3,3-dimethyl)butylamino-doxycycline sulfate

[00038] To a solution of 7-amino-doxycycline (100 mg, 0.2 mmol) in 6 mL of ethylene glycol monomethyl ether were added 0.5 mL of 3,3-dimethyl butyraldehyde, 0.05 mL of concentrated sulfuric acid and 100 mg of 10% Pd/C. The resulting mixture was hydrogenated at atmospheric pressure for 4 hrs, then the catalyst was 5 filtered through celite, washing with methanol. The residue was concentrated and poured into 100 ml cold ether with stirring. The solid that separated was filtered out and dried under vacuum, and 122 mg of product was obtained.

7-Dihexylamino-doxycycline sulfate

[00039] To a solution of 7-amino-doxycycline (100 mg, 0.2 mmol) in 6 mL of ethylene glycol monomethyl ether were added 0.6 mL of hexanal, 0.05 mL of concentrated sulfuric acid and 100 mg of 10% Pd/C. The resulting mixture was hydrogenated at atmospheric pressure for 4 hours, then the catalyst was filtered through celite, washing with methanol. The residue was concentrated and poured into 15 100 ml cold ether with stirring. The solid that separated was filtered out and dried under vacuum, and 129 mg of product was obtained.

7-Isopropylamino-doxycycline sulfate

[00040] To a solution of 7-amino-doxycycline (90 mg, 0.2 mmol) in 8 mL of ethylene glycol monomethyl ether were added 0.5 mL of isobutyraldehyde, 0.05 mL of concentrated sulfuric acid and 90 mg of 10% Pd/C. The resulting mixture was hydrogenated at atmospheric pressure for 6 hours, then the catalyst was filtered through celite, washing with methanol. The residue was concentrated and poured into 100 ml cold ether with stirring. The solid that separated was filtered out and dried 25 under vacuum, and 142 mg of product was obtained.

7-Methyl-7-isopropylamino-doxycycline sulfate

[00041] To a solution of 7-isopropylamino-doxycycline (60 mg) in 4 mL of ethylene glycol monomethyl ether were added 0.4 mL of a 37% aqueous solution of 30 formaldehyde, 0.05 mL of concentrated sulfuric acid and 50 mg of 10% Pd/C. The resulting mixture was hydrogenated at atmospheric pressure for 2 hours, then the catalyst was filtered through celite, washing with methanol. The residue was concentrated and poured into 80 ml cold ether with stirring. The solid that separated was filtered out and dried under vacuum, and 52 mg of product was obtained.

7-Cyclobutylamino-doxycycline sulfate

[00042] To a solution of 7-amino-doxycycline (80 mg) in 7 mL of ethylene glycol monomethyl ether were added 0.3 mL of cyclohexanone, 0.04 mL of concentrated sulfuric acid and 60 mg of 10% Pd/C. The resulting mixture was hydrogenated at atmospheric pressure for 24 hours, then the catalyst was filtered through celite, washing with methanol. The residue was concentrated and poured into 100 ml cold ether with stirring. The solid that separated was filtered out and dried under vacuum, and 85 mg of product was obtained.

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7-Methyl-7-cyclobutylamino-doxycycline sulfate

[00043] To a solution of 7-cyclobutylamino-doxycycline (40 mg) in 3.5 mL of ethylene glycol monomethyl ether were added 0.3 mL of a 37% aqueous solution of formaldehyde, 0.03 mL of concentrated sulfuric acid and 40 mg of 10% Pd/C. The resulting mixture was hydrogenated at atmospheric pressure for 6 hrs, then the catalyst was filtered through celite, washing with methanol. The residue was concentrated and poured into 80 ml cold ether with stirring. The solid that separated was filtered out and dried under vacuum, and 35 mg of product was obtained.

20 **7-Acetamido-doxycycline sulfate**

[00044] To a stirred solution of 7-amino-doxycycline (200 mg) in 3 mL of 1,3-dimethyl-2-imidazolidinone were added 200 mg sodium bicarbonate followed by 0.09 mL of acetyl chloride. The mixture was stirred at room temperature for 1 hr and then filtered. The filtrate was concentrated and poured into 200 ml cold ether with stirring. The product which precipitated was filtered out and dried under vacuum. Obtained 202 mg of product.

7-acetamido-7-isopropyl-doxycycline sulfate

[00045] To a stirred solution of 7-isobutylamino-doxycycline (60 mg) in 1.5 mL of 1,3-dimethyl-2-imidazolidinone were added 60 mg sodium bicarbonate followed by 0.05 mL of acetyl chloride. The mixture was stirred at room temperature for 1 hr 30 min and then filtered. The filtrate was concentrated and poured into 80 ml cold ether with stirring. The product which precipitated was filtered out and dried under vacuum.

7-Diazonium-doxycycline hydrochloride or tetrafluoroborate

[00046] To a stirred solution of 7-amino-doxycycline (200 mg, 0.4 mmol) in 4 mL of 0.1N hydrochloric acid in methanol cooled in an ice bath was added 0.2 mL of n-butyl nitrite. The solution was stirred at 0 °C for 30 minutes, then poured into 200 mL of cold ether with stirring. The solid that separated was filtered out and dried under vacuum, and 173 mg of product was obtained. The diazonium tetrafluoroborate salt was prepared by using tetrafluoroboric acid (54% solution in ether) instead of hydrochloric acid.

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7-Azido-doxycycline

[00047] To a stirred solution of 7-diazonium-doxycycline hydrochloride (80 mg, 0.14 mmol) in 4.5 mL of 0.1N hydrochloric acid in methanol cooled on an ice bath was added 12 mg of sodium azide. The solution was stirred at 0 °C for 2 hours, then 15 poured into 100 mL of cold ether with stirring. The solid that separated was filtered out and dried under vacuum, and 43 mg of product was obtained.

7-Amino-9-nitro-doxycycline sulfate

[00048] To a stirred solution of 7-amino-doxycycline (220 g, 0.44 mmol) in 4 mL of concentrated sulfuric acid at 0 °C was added potassium nitrate (52 mg, 0.51 mmol). The resulting mixture was stirred for 15 minutes at 0 °C, then poured into 300 mL cold ether with stirring. The solid that separated was filtered out, washed with cold ether and dried under vacuum. The product was redissolved in methanol, filtered and the filtrate was poured into ether. The solid that separated was filtered out and dried 25 under vacuum, and 222 g of product was obtained.

7-Dimethylamino-9-nitro-doxycycline sulfate

[00049] To a stirred solution of 7-dimethylamino-doxycycline (40 g, 0.07 mmol) in 1.5 mL of concentrated sulfuric acid at 0 °C was added potassium nitrate (12 mg, 0.12 mmol). The resulting mixture was stirred for 30 min at 0 °C, then poured into 60 mL cold ether with stirring. The solid that separated was filtered out, washed with cold ether and dried under vacuum. LC/MS shows mostly one product containing two nitro groups (possibly a nitrate ester of the desired product).

7-Dimethylamino-9-diazonium-doxycycline sulfate hydrochloride

[00050] To a stirred solution of 7-dimethylamino-9-amino-doxycycline (50 mg) in 1.5 mL of 0.1N hydrochloric acid in methanol cooled in an ice bath was added 0.05 mL of a n-butyl nitrite. The solution was stirred at 0 °C for 30 minutes, then poured 5 into 80 mL of cold ether with stirring. The solid that separated was filtered out and dried under vacuum, and 38 mg of product was obtained.

7-Acetamido-9-nitro-doxycycline sulfate

[00051] To a stirred solution of 7-acetamino-doxycycline (60 g, 0.095 mmol) in 2 10 mL of concentrated sulfuric acid at 0 °C was added potassium nitrate (11 mg, 0.11 mmol). The resulting mixture was stirred for 5 minutes at 0 °C, then poured into 80 mL cold ether with stirring. The solid that separated was filtered out, washed with cold ether and dried under vacuum.

15 **7-Acetamido-9-amino-doxycycline sulfate**

[00052] To a stirred solution of 7-acetamido-9-nitro-doxycycline sulfate (77 mg, 0.12 mmol) in 7 mL of ethylene glycol monomethyl ether was added 60 mg of 10% Pd/C. The reaction mixture was hydrogenated at atmospheric pressure for 7 hours. The catalyst was filtered off through celite washing with methanol. The filtrate was 20 concentrated, then added dropwise to 100 mL cold ether with stirring. The solid that separated was filtered out, washed with cold ether and dried under vacuum, and 62 mg of product was obtained.

7-Acetamido-9-diazonium-doxycycline sulfate hydrochloride

25 [00053] To a stirred solution of 7-acetamido-9-amino-doxycycline (100 mg) in 3 mL of 0.1N hydrochloric acid in methanol cooled in an ice bath was added 0.1 mL of n-butyl nitrite. The solution was stirred at 0 °C for 30 minutes, then poured into 100 mL of cold ether with stirring. The solid that separated was filtered out and dried under vacuum, and 92 mg of product was obtained.

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7-Acetamido-9-azido-doxycycline sulfate

[00054] To a stirred solution of 7-acetamido-9-diazonium-doxycycline (112 mg, 0.19 mmol) in 6 mL of 0.1N hydrochloric acid in methanol cooled in an ice bath was added 14 mg (0.21 mmol) of sodium azide. The solution was stirred at 0 °C for 35

minutes, then poured into 150 mL of cold ether with stirring. The solid that separated was filtered out and dried under vacuum, and 94 mg of product was obtained.

Doxycycline methiodide

5 [00055] Doxycycline free base (315 mg) was dissolved in 2 mL of methanol and 10 mL of THF, then 1 mL of methyl iodide was added. The reaction mixture was kept for 5 days; no crystallization of the product occurred. The reaction mixture was concentrated and poured into 300 mL of cold ether with stirring. The product that separated was filtered out and dried under vacuum, and 269 mg was obtained; LC/MS shows pure product.

5-Acetoxy-doxycycline

15 [00056] A solution of doxycycline (100 mg) in 3 mL of a 30% HBr in acetic acid solution was stirred at room temperature for 3.5 days; the reaction mixture was then poured into 100 mL of cold ether with stirring and the product that separated was filtered out. LC/MS showed incomplete conversion of starting material, so the product (86 mg) was resubjected to the reaction conditions for 9 hrs, and then isolated in the same way.

20 9-*tert*-butyl-doxycycline

[00057] A solution of doxycycline (100 mg) in 2 mL of *tert*-butanol and 3 mL of methanesulfonic acid was stirred at room temperature for 18 hours, then poured into 100 mL of cold water and extracted with n-butanol. The organic extracts were basified with a 1N sodium hydroxide solution and the pH was quickly adjusted to 6.5-25 7 with concentrated hydrochloric acid. A solid precipitated which was filtered out, then dissolved in methanol, concentrated and added dropwise to 30 mL of an ether/PET ether cold solution with stirring. The solid that separated was filter out and dried under vacuum.

30 9-*tert*-butyl-7-nitro-doxycycline

[00058] A solution of doxycycline hydrochloride (2 g) in 10 mL of *tert*-butanol and 30 mL of methanesulfonic acid was stirred at room temperature for 3 hours, then 500 mg of potassium nitrate was added and the resulting mixture stirred for an additional hour. The reaction mixture was poured in 100 ml cold solution of water containing 23

g of sodium hydroxide; more dilute NaOH solution was added dropwise until the reaction mixture became basic. The pH was quickly adjusted to 6.5-7 with concentrated hydrochloric acid. The gluey dark solid that separated was filtered out. The aqueous filtrate was extracted once with n-butanol to recover more product. The 5 crude product was dissolved in the minimum amount of methanol and purified by HPLC. The combined fractions from HPLC were concentrated to dryness, dissolved in methanol and added dropwise to 50 mL of a 3:1 mixture of ether/PET ether. The solid that separated was filtered out, and 788 mg of pale yellow product was obtained.

10 *9-tert-butyl-7-amino-doxycycline*

[00059] To a solution of *9-tert-butyl-7-doxycycline* (500 mg) in 25 mL of ethylene glycol monomethyl ether was added 350 mg of 10% Pd/C and the resulting mixture was hydrogenated at atmospheric pressure for 17 hours. The catalyst was filtered through celite, washing with methanol. The residue was concentrated, dissolved in 15 THF and added dropwise to a cold 1:1 solution of ether/PET ether with stirring. The product that precipitated was filtered out and dried under vacuum, and 452 mg of product was obtained.

9-*tert*-butyl-7-diazonium-doxycycline

20 [00060] To a stirred solution of *9-tert-butyl-7-amino-doxycycline* (93 mg, 0.18 mmol) in 2.5 mL of 0.1N hydrochloric acid in methanol cooled in an ice bath was added 0.1 mL of n-butyl nitrite. The solution was stirred at 0 °C for 30 minutes, and then half was poured into 30 mL of a 3:1 cold mixture of ether/PET ether with stirring. The product stuck to the bottom of the beaker as a gum. It was redissolved 25 in methanol and concentrated, the residue was poured into 20 mL of cold ether and the solid that separated was filtered out immediately and dried under vacuum; 42 mg of product was obtained. The other half of the reaction mixture was used directly for the *9-tert*-butyl material below.

30 *9-tert-butyl-7-azido-doxycycline*

[00061] To a stirred solution of *9-tert-butyl-7-diazonium-doxycycline* reaction mixture (1.5 mL) at 0 °C were added 8 mg (0.12 mmol) of sodium azide. The solution was stirred for 3 hours while allowed to warm to room temperature. After concentration of the reaction mixture, the residue was poured into a cold 3:1 mixture

of ether/PET ether with stirring. No solid separated, so the solution was concentrated to dryness to obtain 35 mg of product.

9-*tert*-butyl-7-dimethylamino-doxycycline

5 **[00062]** To a stirred reaction mixture from 9-*tert*-butyl-7-amino-doxycycline containing 167 mg of 9-*tert*-butyl-7-amino-doxycycline in 10 mL of ethylene glycol monomethyl ether and 115 mg of 10% Pd/C was added 1.2 ml of a 37% aqueous solution of formaldehyde. The resulting mixture was hydrogenated for 3.5 hours at atmospheric pressure, then the catalyst was filtered through celite washing with
10 methanol. The filtrate was concentrated and the residue was poured into 10 mL of cold ether with stirring. The product that separated was filtered out and dried under vacuum. LC/MS showed the expected product, but was contaminated by baseline impurity.

15 **9-*tert*-butyl-7-acetamido-doxycycline**

[00063] To a stirred solution of 9-*tert*-butyl-7-amino-doxycycline (100 mg) in 2 mL of 1,3-dimethyl-2-imidazolidinone were added 100 mg of sodium bicarbonate followed by 0.07 mL of acetyl chloride. The mixture was stirred at room temperature for 1.5 hours and then filtered. The filtrate was concentrated and poured into 100 mL of cold ether with stirring. The product that separated was filtered out. The crude product was redissolved in methanol, filtered and added dropwise to a cold 3:1 mixture of ether/PET ether with stirring. The product was collected by filtration and dried under vacuum, and 76 mg of product was obtained.

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4-dedimethylamino-doxycycline

[00064] To a stirred solution of tetracycline methiodide (860 mg) in 12 mL of acetic acid and 12 mL of water were added 432 mg of zinc dust. After stirring for 15
30 min the zinc powder was filtered out. The filtrate was diluted with 86 mL of water containing 0.86 mL of concentrated hydrochloric acid. The product that separated was filtered out and dried under vacuum, and 419 mg of product was obtained.

4-dedimethylamino-9-nitro-doxycycline

[00065] To a stirred solution of 4-dedimethylamino-doxycycline (402 mg, 1 mmol) in 20 mL of concentrated sulfuric acid at 0 °C was added 111 mg of potassium nitrate. After stirring for 20 minutes, the reaction mixture was poured into 100 mL of cold water. n-Butanol was added to the water containing the product and the organic layer 5 was separated. Extraction with n-butanol was repeated twice. The combined organic extracts were concentrated and added to cold water with stirring. The product that separated was filtered out; more product was recovered by re-extracting the aqueous filtrate with n-butanol, concentrating, pouring into cold water and filtering. After drying under vacuum, 353 mg of product were obtained.

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4-dedimethylamino-9-amino-doxycycline

[00066] To a stirred solution of 4-dedimethylamino-9-nitro-doxycycline (353 mg) in 17 mL of ethylene glycol monomethyl ether were added 0.1 mL of concentrated sulfuric acid and 200 mg of 10% Pd/C, and the resulting mixture was hydrogenated at 15 atmospheric pressure for 10 hours. The catalyst was filtered through celite, washing with methanol. The filtrate was concentrated and poured into 100 mL of ether. The solid that separated was filtered out and dried under vacuum, and 292 mg of product was obtained.

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4-dedimethylamino-9-diazonium-doxycycline hydrochloride

[00067] To a stirred solution of 4-dedimethylamino-9-amino-doxycycline (220 mg) in 5 mL of 0.1N hydrochloric acid in methanol cooled in an ice bath was added 0.2 mL of n-butyl nitrite. The solution was stirred at 0 °C for 30 minutes, then poured into 150 mL of a 2:1 cold mixture of ether/PET ether with stirring. The solid that 25 separated was filtered out and dried under vacuum, and 191 mg of product was obtained.

4-dedimethylamino-9-azido-doxycycline

[00068] To a stirred solution of 4-dedimethylamino-9-diazonium-doxycycline (150 mg, 0.32 mmol) in 6.5 mL of 0.1N hydrochloric acid in methanol cooled in an ice 30 bath were added 23 mg of sodium azide, and the resulting mixture was stirred for 1 hour at 0 °C. The mixture was then poured into 120 mL of cold ether with stirring. Some solid formed, which was filtered out and found not to be the expected product. The filtrate was concentrated to dryness, taken up in methanol and poured into 50 mL

of cold water with stirring. The solid that separated was filtered out and dried under vacuum; more product was obtained by extracting the filtrate with n-butanol, concentrating, pouring into water and filtering out the solid, and 84 mg of product was obtained.

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4-dedimethylamino-7-nitro-9-*tert*-butyl-doxycycline

[00069] A solution of 4-dedimethylamino-doxycycline (500 mg 1.25 mmol) in 3 mL of *tert*-butanol and 15 mL of methanesulfonic acid was stirred at room temperature for 15 hours, then 140 mg of potassium nitrate was added and the 10 resulting mixture stirred for an additional hour. The reaction mixture was poured in 200 ml of cold water with stirring. The solid that separated was filtered out and air dried. The crude product was dissolved in the minimum amount of methanol and purified by HPLC. The combined fractions from HPLC were concentrated to dryness, dissolved in methanol and added dropwise to 50 mL of cold water. The solid that 15 separated was filtered out and dried under vacuum, and 217 mg of product was obtained.

4-dedimethylamino-7-amino-9-*tert*-butyl-doxycycline

[00070] To a stirred solution of 4-dedimethylamino-7-nitro-9-*tert*-butyl-doxycycline (217 mg, 0.42 mmol) in 12 mL of ethylene glycol monomethyl ether were added 170 mg of 10% Pd/C, and the resulting mixture was hydrogenated at atmospheric pressure for 13 hours. The catalyst was filtered through celite, washing with methanol. The filtrate was concentrated and poured into 20 of a 3:1 mixture of ether/PET ether, and 40 mg of product was obtained. The orange filtrate was 25 concentrated to dryness to obtain additional 138 mg of product.

4-dedimethylamino-7-diazonium-9-*tert*-butyl-doxycycline

[00071] To a stirred 4-dedimethylamino-7-amino-9-*tert*-butyl-doxycycline (165 mg) in 5 mL of 0.1N hydrochloric acid in methanol cooled in an ice bath was added 0.15 mL of n-butyl nitrite. The solution was stirred at 0 °C for 30 minutes; half of the material was poured into 50 mL of cold ether with stirring. The solid that separated was filtered out and dried under vacuum, and 50 mg of product was obtained.

4-dedimethylamino-7-amino-doxycycline

[00072] To a stirred solution of 4-dedimethylamino-doxycycline (400 mg) in 4 mL of THF and 4.5 mL of methanesulfonic acid at 0 °C were added 418 mg (1.4 mmol) of dibenzyl azodicarboxylate, and the resulting mixture was stirred for 4 hours while warming to room temperature. Water and n-butanol were added and two layers separated. The aqueous layer was extracted with a mixture of ether/n-butanol. The combined organic extracts were concentrated to dryness, and 10 mL of ethylene glycol monomethyl ether were added. Then, 430 mg of 10% Pd/C were added and the resulting mixture hydrogenated at atmospheric pressure for 12 hours. The catalyst was filtered off through celite, washing with methanol. The filtrate was concentrated and poured into a 3:1 cold mixture of ether/PET ether with stirring; the product that separated was filtered out and dried under vacuum, and 114 mg of product was obtained.

15 **9-nitro-minocycline sulfate**

[00073] To a stirred solution of minocycline hydrochloride (1 g, 2.02 mmol) in 30 mL of concentrated sulfuric acid at 0 °C was added potassiumnitrate (246 mg). The resulting mixture was stirred for 45 minutes at 0 °C, then poured into 1.2 L cold ether with stirring. The solid that separated was filtered out, washed with cold ether and dried under vacuum, and 1.33 g of product was obtained.

9-amino-minocycline sulfate

[00074] To a stirred solution of 9-nitro-minocycline sulfate (500 g, 0.83 mmol) in 10 mL of ethylene glycol monomethyl ether and 7 mL of methanol were added 250 mg of 10% Pd/C. The reaction mixture was hydrogenated at atmospheric pressure for 4 hours. The catalyst was filtered off through celite, washing with methanol. The filtrate was concentrated, then added dropwise to 600 mL cold ether with stirring. The solid that separated was filtered out, washed with cold ether and dried under vacuum, and 465 mg of product was obtained.

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9-diazonium-minocycline sulfate hydrochloride

[00075] To a stirred solution of 9-amino-minocycline sulfate (100 mg) in 3 mL of 0.1N hydrochloric acid in methanol cooled in an ice bath was added 0.1 mL of tert-butyl nitrite (or n-butyl nitrite). The solution was stirred at 0 °C for 30 minutes, then

poured into 100 mL of cold ether with stirring. The solid that separated was filtered out and dried under vacuum, and 87 mg of an orange-brown product was obtained.

9-azido-minocycline sulfate

5 [00076] To a stirred solution of 9-diazonium-minocycline sulfate hydrochloride (485 mg, 0.83 mmol) in 18 mL of 0.1N hydrochloric acid in methanol were added 59 mg of sodium azide. The solution was stirred at room temperature for 2 hours, then filtered, and the filtrate was poured into 500 mL of cold ether with stirring. The solid that separated was filtered out and dried under vacuum, and 480 mg of beige product
10 was obtained.

9-acetamido-minocycline

[00077] To a stirred solution of 9-amino-minocycline sulfate (100 mg, 0.175 mmol) in 1.5 mL of 1,3-dimethyl-2-imidazolidinone were added 100 mg of sodium bicarbonate followed by 0.05 mL of acetyl chloride. The mixture was stirred at room temperature for 45 minutes and then poured into 150 mL of cold ether with stirring. The solid that separated was filtered out and dried under vacuum, and 165 mg of product was obtained.

20 4-dedimethylamino-minocycline

[00078] Minocycline free base (175 mg, 0.38 mmol) was dissolved in 2 mL of THF, then 0.43 mL of methyl iodide was added. No crystallization of the product occurred. The reaction mixture was concentrated and poured into 200 mL of cold ether/PET ether 40:60 with stirring. The product that separated was filtered out and dried under vacuum; the product was resubjected to the reaction conditions for 3 additional days, then precipitated from ether as before, and 123 mg of minocycline methiodide was obtained. The crude product was dissolved in 2 mL of acetic acid, and 2 mL of water and 60 mg of zinc dust were added with stirring. After 15 minutes, the zinc powder was filtered out. The filtrate was diluted with 15 mL of water containing concentrated hydrochloric acid. The combined organic extracts were dried over magnesium sulfate and concentrated. The solid that separated was filtered out and dried under vacuum, and 150 mg of a yellow product was obtained.

4-dedimethylamino-9-nitro-minocycline

[00079] 4-Dedimethylamino minocycline (415 mg, 1 mmol) was dissolved in 30 mL of concentrated sulfuric acid at room temperature. The solution was cooled on an ice bath and potassium nitrate (110 mg, 1.09 mmol) was added with stirring. The resulting mixture was stirred for 15 minutes, then poured into 300 mL cold ether with 5 stirring. The solid that separated was filtered out, washed with cold ether and dried under vacuum. LC/MS showed incomplete conversion of starting material so the product was redissolved in 15 mL of concentrated sulfuric acid, 50 mg of potassium nitrate were added at 0 °C and the resulting mixture was stirred for 45 minutes. The product was isolated as before, and 542 mg of product was obtained.

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7,9-dibromo sencycline

[00080] To a stirred solution of sencycline (50 mg, 0.12 mmol) in 1.5 mL of concentrated sulfuric acid at 0 °C were added 42 mg of N-bromosuccinimide. After 30 minutes, the reaction mixture was poured into 60 mL of cold ether with stirring. 15 The solid that separated was filtered out and dried under vacuum, and 70 mg of yellow product was obtained.

Mix of 7-nitro and 9-nitro-sencycline

[00081] To a stirred solution of sencycline (40 mg, 0.09 mmol) in 1.5 mL of concentrated sulfuric acid at 0 °C was added 10 mg of potassium nitrate. After 20 20 minutes, the reaction mixture was poured into 50 mL of cold ether with stirring. The solid that separated was filtered out and dried under vacuum, and 59 mg of product was obtained. LC/MS shows a 1.6:1 ratio of two mono-nitrated products.

[00082] In further testing of the present invention, the Minimum Inhibitory Concentrations (MIC) of five test products when challenged with ten different microorganism strains were evaluated. The study was an MIC evaluation for five test products, performed using a modification of the Macrodilution Broth Method outlined in NCCLS document M7-A5, *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically*, Fifth Edition. Each test product was evaluated, in 25 duplicate, against challenge suspensions of ten microorganism strains. Prior to using each product for testing, it was diluted (w/v) in sterile Mueller-Hinton Broth (MHB) to achieve an initial concentration, based on a potency of 1000 µg/mg, of 160 µg/mL.

Product 1 - Tetracycline Derivative
Lot Number: INN01182 (MW 615)

14.8 mg of this product was dissolved in 92.5 mL of MHB to achieve the initial concentration of 160 μ g/mL

Product 2 - Tetracycline Derivative
Lot Number: INN01183 (MW 627)

13.6 mg of this product was dissolved in 85.0 mL of MHB to achieve the initial concentration of 160 μ g/mL

Product 3 - Tetracycline Derivative
Lot Number: INN01185 (MW 655)

11.9 mg of this product was dissolved in 74.4 mL of MHB to achieve the initial concentration of 160 μ g/mL

Product 4 - Tetracycline Derivative
Lot Number: INN01189 (MW 625)

9.6 mg of this product was dissolved in 60.0 mL of MHB to achieve the initial concentration of 160 μ g/mL

Product 5 - Tetracycline Derivative
Lot Number: INN01195 (MW 643)

13.8 mg of this product was dissolved in 86.25 mL of MHB to achieve the initial concentrations of 160 μ g/mL

- [00083] Approximately 48 hours prior to testing, separate sterile tubes of Tryptic Soy Broth were inoculated from lyophilized vials containing each of the challenge microorganisms, as found in Table I, below. The broth cultures were incubated at 35° ± 2°C for approximately 24 hours. The broth cultures prepared as described above were inoculated onto the surface of Tryptic Soy Agar contained in Petri plates, and incubated at 35° ± 2°C for approximately 24 hours. This produced “lawns” of the microorganisms on the surface of the agar plates, which were used to prepare the challenge suspensions.
- [00084] Prior to initiating testing, an initial challenge suspension containing approximately 1.0×10^9 CFU/mL was prepared for each microorganism by inoculating a test tube of Sodium Chloride Irrigation with microorganisms taken from the plates of solid media, prepared as described above. The final challenge suspensions containing approximately 1.0×10^6 CFU/mL were prepared for each microorganism species, by placing a 0.2 mL aliquot of the 1.0×10^9 CFU/mL suspension into a sterile 250 mL polypropylene bottle, containing 200 mL of Mueller-Hinton Broth. The final challenge suspensions were mixed thoroughly prior to use in testing. The final plated dilutions were 10^{-3} , 10^{-4} and 10^{-5} . These plates were incubated at 35° ± 2°C until sufficient growth was observed (Table I).

[00085] Following incubation, the colonies on the plates were counted manually using a hand-tally counter. Counts in the 30 to 300 CFU range were used in the data calculations.

The Initial Population (CFU/mL) was calculated for each challenge suspension as
5 follows:

$$\text{CFU/mL} = (C_i \times 10^{-D})$$

Where:

C_i = Average of the 2 Plates Counted

D = Dilution Factor of the Plate Counts Used

10 The population (CFU/mL) per tube of product/broth following inoculation was calculated for each challenge suspension as follows:

$$\text{Population per Tube (CFU/mL)} = \frac{(C_i \times 10^{-D})}{2}$$

Where:

15 C_i = Average of the Two (2) Plates Counted

D = Dilution Factor of the Plate Counts Used

2 $=$ Total Volume (mL) present in each product/broth tube following inoculation

20 [00086] The testing procedure was as follows: 30 mL aliquots of Mueller-Hinton Broth were dispensed into sterile bottles. A 30 mL aliquot of product (initial concentration of 160 $\mu\text{g}/\text{mL}$) was dispensed into the first of a series of 11 sterile bottles, each containing 30 mL of Mueller-Hinton Broth, and mixed thoroughly in order to achieve the 1:2 (v/v) dilution of product. A 30 mL aliquot was removed from
25 the bottle prepared as described above, and used to initiate a 1:2 (v/v) dilution series through the remaining 10 bottles (1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, 1:512, 1:1,024 and 1:2,048 product dilutions), each containing 30 mL of Mueller-Hinton Broth. Each bottle was mixed thoroughly prior to removing the 30 mL aliquot required for the next dilution in the series. 1.0 mL aliquots of each product dilution
30 prepared and 1.0 mL aliquots of the initial product preparation (160 $\mu\text{g}/\text{mL}$ product concentration) were transferred to separate sterile test tubes. A series of 12 tubes, each containing 1.0 mL of the appropriate product dilution (1:1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, 1:512, 1:1,024, and 1:2,048) was prepared for each

microorganism evaluated against each product (Table I). This resulted in product concentrations ranging from 160 $\mu\text{g}/\text{mL}$ to 0.078 $\mu\text{g}/\text{mL}$.

[00087] A 1.0 mL aliquot of a suspension containing approximately 1.0×10^6 CFU/mL was introduced into each product dilution tube in the series, thereby resulting in a final product dilution series of 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, 1:512, 1:1,024, 1:2,048, and 1:4,096, with each dilution tube containing approximately 5.0×10^5 CFU/mL of the challenge microorganism. This resulted in final product concentrations ranging from 80 $\mu\text{g}/\text{mL}$ to 0.039 $\mu\text{g}/\text{mL}$. The test procedure described above was performed, in duplicate, for each microorganism species tested (Table I) against each test product. A positive control tube (growth control) containing a 1.0 mL aliquot of Mueller-Hinton Broth and a 1.0 mL aliquot of the challenge suspension was prepared for each microorganism (Table I). A negative (media) control tube (sterility control; no microbial inoculation) of Mueller-Hinton Broth was also prepared.

[00088] Product turbidity controls were prepared for each product by transferring 1.0 mL aliquots of each product dilution into separate sterile test tubes. A 1.0 mL aliquot of sterile Mueller-Hinton Broth was introduced into each product dilution tube created and mixed thoroughly using a vortex mixer, thereby resulting in a final product dilution series identical to that described above. The challenge suspension/product dilution tubes and the controls were incubated at $35^\circ \pm 2^\circ\text{C}$ for 20 hours, until good growth was apparent in the positive control tubes.

[00089] Following incubation, the tubes were examined for growth of the microorganisms, determined visually on the basis of turbidity.

[00090] The Minimum Inhibitory Concentration (MIC) for each test product versus each challenge microorganism was recorded as the highest dilution of test product that completely inhibited growth of the microorganism, as detected by the unaided eye. The results of the duplicate runs for each test product versus each microorganism were recorded and then averaged together to provide the final reported values.

TABLE I

Microorganism Species	ATCC #	Incubation Time* (MIC Tubes)	Incubation Temp.*	Media
Enterococcus faecalis	29212	20 Hours	$35^\circ \pm 2^\circ\text{C}$	TSB/TSA/MHB
Escherichia coli	25922	20 Hours	$35^\circ \pm 2^\circ\text{C}$	TSB/TSA/MHB
Escherichia coli (Tetracycline-	51657	20 Hours	$35^\circ \pm 2^\circ\text{C}$	TSB/TSA/MHB

Resistant)				
Escherichia coli (Tetracycline-Resistant)	51658	20 Hours	35° ± 2°C	TSB/TSA/MHB
Escherichia coli (Tetracycline-Resistant)	51659	20 Hours	35° ± 2°C	TSB/TSA/MHB
Staphylococcus aureus (Tetracycline-Resistant)	27217	20 Hours	35° ± 2°C	TSB/TSA/MHB
Staphylococcus aureus (Tetracycline-Resistant)	27659	20 Hours	35° ± 2°C	TSB/TSA/MHB
Staphylococcus aureus (Tetracycline-Resistant)	27660	20 Hours	35° ± 2°C	TSB/TSA/MHB
Staphylococcus aureus	29213	20 Hours	35° ± 2°C	TSB/TSA/MHB

* = Initial Population plates were inadvertently incubated at room temperature (20° - 27°C) for approximately 48 hours prior to being incubated at 35° ± 2°C for approximately 72 hours.

- 5 [00091] Table II presents the Minimum Inhibitory Concentrations, expressed as product dilution, for each test product versus each of the 10 microorganisms tested. Table III presents the Minimum Inhibitory Concentrations, expressed as product concentration ($\mu\text{g/mL}$), for each test product versus each of the 10 microorganisms tested. The initial solution prepared for each product had a concentration, based on a 10 potency of 1000 $\mu\text{g}/\text{mg}$, of 160 $\mu\text{g/mL}$.

TABLE II

Microorganism Species	ATCC #	Minimum Inhibitory Concentrations (Expressed as Product Dilution)				
		Product #1	Product #2	Product #3	Product #4	Product #5
Enterococcus faecalis	29212	< 1 : 2	1 : 16	1 : 16	1 : 2	1 : 8
Escherichia coli	25922	1 : 2	< 1 : 2	< 1 : 2	< 1 : 2	1 : 2
Escherichia coli (Tetracycline-Resistant)	51657	< 1 : 2	< 1 : 2	< 1 : 2	< 1 : 2	< 1 : 2
Escherichia coli (Tetracycline-Resistant)	51658	< 1 : 2	< 1 : 2	< 1 : 2	< 1 : 2	< 1 : 2
Escherichia coli (Tetracycline-Resistant)	51659	< 1 : 2	< 1 : 2	< 1 : 2	< 1 : 2	< 1 : 2
Pseudomonas aeruginosa	27853	< 1 : 2	< 1 : 2	< 1 : 2	< 1 : 2	< 1 : 2
Staphylococcus aureus (Tetracycline-Resistant)	27217	< 1 : 2	1 : 32	1 : 32	1 : 8	1 : 16
Staphylococcus aureus (Tetracycline-Resistant)	27659	< 1 : 2	1 : 32	1 : 24	1 : 8	1 : 16
Staphylococcus aureus (Tetracycline-Resistant)	27660	< 1 : 2	1 : 32	1 : 16	1 : 8	1 : 32
Staphylococcus aureus	29213	1 : 8	1 : 32	1 : 32	1 : 8	1 : 32

TABLE III

Microorganism Species	ATCC #	Minimum Inhibitory Concentrations (Expressed as Product Dilution)				
		Product #1	Product #2	Product #3	Product #4	Product #5
Enterococcus faecalis	29212	> 80	10	10	80	20

<i>Escherichia coli</i>	25922	80	> 80	> 80	> 80	80
<i>Escherichia coli</i> (Tetracycline-Resistant)	51657	> 80	> 80	> 80	> 80	> 80
<i>Escherichia coli</i> (Tetracycline-Resistant)	51658	> 80	> 80	> 80	> 80	> 80
<i>Escherichia coli</i> (Tetracycline-Resistant)	51659	> 80	> 80	> 80	> 80	> 80
<i>Pseudomonas aeruginosa</i>	27853	> 80	> 80	> 80	> 80	> 80
<i>Staphylococcus aureus</i> (Tetracycline-Resistant)	27217	> 80	5	5	20	10
<i>Staphylococcus aureus</i> (Tetracycline-Resistant)	27659	> 80	5	6.67	20	10
<i>Staphylococcus aureus</i> (Tetracycline-Resistant)	27660	> 80	5	10	20	5
<i>Staphylococcus aureus</i>	29213	20	5	5	20	5

[00092] In additional studies, the activity of the tetracycline derivatives of the present invention as inhibitors of matrix degrading metalloproteinases (MMPs) that are involved in malignant tumor growth and metastasis were investigated. The 5 studies used the Dunning MAT LyLu tumor model which was developed in Copenhagen rats from a transplantable tumor, transferred from an original prostate tumor from the dorsal prostate of an aged Copenhagen male rat. The MAT LyLu tumor is spontaneously metastatic to lymph nodes and lungs when injected, and produces bone metastases in approximately 80–100% of the recipient animals. Fresh 10 dosing solutions containing 10 mg/ml of each test article in 2% carboxymethylcellulose were prepared daily and used in the testing.

[00093] The studies initially evaluated toxicity in two groups of five non-tumor bearing animals, each receiving the two tetracycline analogs (9-amino-doxycycline and 9-nitro-doxycycline) at a dose of 40 mg/kg daily for seven days.

15

[00094] Three groups of animals (10/group) were dosed by gavage with either vehicle, or one of the two analogs daily for three weeks, and then three times per week until death or sacrifice. Dosing was begun seven days prior to implantation of the MAT LyLu tumor cells by tail vein injection. Approximately 0.1 ml of tumor cell 20 suspension was injected into one of the lateral tail veins. The dose to be chosen was based on two factors: (1) efficacy and (2) morbidity associated with the analog, if any. The following table (Table IV below), demonstrates survival time and weekly body weight of animals in control and treated groups. As shown in the table, 9-amino-doxycycline and 9-nitro-doxycycline demonstrated a marked increase in 25 surviving number after tumor implantation, while also resulting in indistinguishable

body weight changes as compared to the control group. This latter finding is particularly significant, in that conventional anti-cancer treatments often result in significant weight loss.

TABLE IV

Number of Days Post Tumor Implantation	Surviving Number		
	9-amino-doxycycline	9-nitro-doxycycline	Control
14	10	8	9
17	10	8	8
24	10	8	7
27	10	8	6
28	9	8	6
29	9	6	5
30	7	4	5
34	7	4	3
42	7	4	3

5

[00095] In further studies, the inhibitory activity of MMPs, as well as percent inhibition of various cancers by various tetracycline derivative were evaluated. Results appear in Table V, below, and demonstrate the efficacy of the treatments of the present invention. One objective of the studies was to assess a series of 10 tetracycline derivatives as inhibitors of purified human matrix metalloproteinases including MMP1, 2, 3, 7, 9 and 14. The tetracycline derivatives were effective inhibitors in the mM- μ M range.

EXPERIMENTAL PROTOCOLS

15 1. Protocol for measuring MMP1 (Collagenase-1) inhibition by tetracycline derivatives

MMP1 activity was determined by the release of soluble ^{14}C labelled collagen fragments from ^{14}C acetylated rat skin type I collagen (Methods in Enzymology 80 20 711, 1981).

Assay constituents:

100 µg ¹⁴C labelled rat skin type I collagen (5-6000 dpm)

50 mM Tris HCl pH 7.9

5 15 mM CaCl₂ 0.02% azide

Human recombinant MMP1 3-4nM

± Tetracycline (up to 1 mM) in a total volume of 300 µl.

[00096] Incubations were at 35°C for 5 hours. Uncleaved collagen fibrils (which form within the first 10 minutes) were removed by centrifugation at 10,000 g, 4°C, 10 minutes. 200 µl supernatant were counted in Packard Opti Phase Supermix scintillation fluid using a scintillation counter. Only data derived from within the linear part of the assay (10 - 70% lysis of the collagen) were utilized. Based on percentage inhibition of the activity of MMP1 alone by a range of concentrations of each sample, the IC₅₀ value for each tetracycline was calculated by linear regression analysis.

2. Protocol for measuring MMP2 (Gelatinase A) inhibition

MMP2 activity was determined by the release of trichloroacetic acid, TCA, soluble fragments from ¹⁴C acetylated rat skin type I gelatin (Methods in Enzymology 248, 470, 1995. Biochem. J. 195, 1981)

Assay constituents:

100 µg ¹⁴C labelled gelatin (type I collagen denatured 60°C 20 min)

25 50 mM Tris HCl pH 7.9

15 mM CaCl₂, 0.02% azide

Human recombinant MMP1, 0.1-0.5 nM

± Tetracycline (up to 1 MM) in a total volume of 250 µl.

30 [00097] Incubations were set at 37°C for 16 hours. The reaction was stopped by cooling the incubations and addition of 50 µl cold 90% (w/v) trichloroacetic acid with careful mixing. TCA precipitation was allowed to proceed for 15 minutes at 4°C prior to centrifugation at 10,000 g for 10 minutes at 4°C. 200 µl of the TCA supernatant was taken for the determination of radioactive content by scintillation

counting (Packard Opti Phase Supermix scintillation fluid). Only data derived from within the linear part of the assay (10 - 70% lysis) were utilized. The percentage inhibition of MMP2 by each tetracycline concentration was plotted to derive the IC₅₀ value, using linear regression analysis.

5

3. Protocol for measuring MMP3 (Stromelysin 1) inhibition

MMP3 activity was determined by the release of trichloracetic acid, TCA, soluble fragments from ¹⁴C acetylated β casein (Sigma) as described in Methods in Enzymology 248, 451 (1995).

10

Assay constituents:

100 μ g ¹⁴C casein (7000 dpm)

50 mM Tris HCl, pH 7.9

15 mM CaCl₂, 0.02% azide

15 Human recombinant MMP3, 0.25-1.0 nM

\pm tetracycline (up to 1 mM) in a total volume of 250 μ l.

[00098] Incubations were at 37°C for 16 hours. The reaction was stopped by the addition of 500 μ l cold 18% TCA and standing on ice for 15 minutes. TCA precipitates were removed by centrifugation at 10,000 g for 10 minutes at 4°C and 200 μ l supernatant was taken for scintillation counting. Only data derived from within the linear part of the assay (10 - 60% lysis) were utilized. The percentage inhibition of MMP3 by each concentration of the candidate tetracycline was plotted to derive the IC₅₀ value, using linear regression analysis.

25

4. Protocol for measuring MMP7 (Matrilysin) inhibition

MMP7 activity was determined by the release of trichloracetic acid, TCA, soluble fragments from ¹⁴C acetylated β casein (Sigma) as described in Methods in Enzymology 248, 451 (1995).

Assay constituents:

100 μ g ¹⁴C casein (7000 dpm)

50 mM Tris HCl, pH 7.9

15 mM CaCl₂, 0.02% azide
Human recombinant MMP7, 1.5nM
± tetracycline (up to 1 mM) in a total volume of 250 µl.

5 [00099] Incubations were at 37°C for 16 hours. The incubation was stopped by the addition of 500 µl cold 18% TCA and standing on ice for 15 minutes. TCA precipitates were removed by centrifugation at 10,000 g for 10 minutes at 4°C, and 200 µl supernatant was taken for scintillation counting. Only data derived from within the linear part of the assay (10 - 60% lysis) were utilized. The percentage 10 inhibition of MMP7 by each concentration of the candidate tetracycline were plotted to derive the IC50 value.

5. Protocol for measuring MMP9 (Gelatinase B) inhibition

15 MMP9 activity was determined by the release of trichloracetic acid, TCA soluble fragments from ¹⁴C acetylated rat skin type I (Methods in Enzymology 248, 470, 1995. Biochem. J. 195, 1981)

Assay constituents:

20 100 µg ¹⁴C labelled gelatin (collagen denatured 60°C 20 min)
50 mM Tris HCl pH 7.9
15 mM CaCl₂, 0.02% azide
Human recombinant MMP9, 1.2 - 2 nM
± Tetracycline (up to 1 mM) in a total volume of 250 µl.

25 [000100] Incubations were at 37°C for 16 hours. The incubations were stopped by cooling the incubations and addition of 50 µl cold 90% (w/v) trichloracetic acid with careful mixing. TCA precipitation was allowed to proceed for 15 minutes at 4°C prior to centrifugation at 10,000 g for 10 minutes at 4°C. 200 µl of the TCA 30 supernatant was taken for the determination of radioactive content by scintillation counting (Packard Opti Phase Supermix scintillation fluid). Only data derived from within the linear part of the assay (10 - 70% lysis) were utilized. The percentage inhibition of MMP9 by each tetracycline concentration were plotted to derive the IC50 value.

6. Protocol for measuring MMP14 (MT1-MMP) inhibition by tetracycline derivatives

MMP14 activity was determined by the release of soluble ¹⁴C labelled collagen
5 fragments from ¹⁴C acetylated rat skin type I collagen (Methods in Enzymology 80
711, 1981).

Assay constituents:

- 100 µg ¹⁴C labelled collagen (5-6000 dpm)
10 50 mM Tris HCL pH 7.9
15 mM CaCl₂ 0.02% azide
Human recombinant MMP14 50-100nM
± Tetracycline (up to 1 mM) in a total volume of 300 µl.
- 15 [000101] Incubations were at 35°C for 15 hours. Uncleaved collagen fibrils (which form within the first 10 minutes) were removed by centrifugation at 10,000 g 4°C 10 minutes 200 µl supernatant were counted in Packard Opti Phase Supermix scintillation fluid using a scintillation counter. Only data derived from within the linear part of the assay (10 - 70% lysis of the collagen) were utilized. Based on 20 percentage inhibition of the activity of MMP1 alone by a range of concentrations of each sample, the IC50 value for each tetracycline was calculated.

[000102] Another objective was to analyze the effects of specific, tetracycline derived compounds on the *in-vitro* chemo-invasive potential of:

- 25 C8161 human melanoma cells
MDA-MB-231 human breast cancer cells
PC3 human prostate cancer cells
RPM18226 human myeloma cancer cells
30 Ark human myeloma cancer cells
Arp-1 human myeloma cancer cells

[000103] The Membrane Invasion Culture System (MICS) *in vitro* chemo-invasion assay was chosen to measure changes in the invasive potential of specific human cancer cells in response to tetracycline derived compounds. Stock solutions of each 35 compound were hydrated in water containing 2% DMSO and pH 10.0. Upon

solubilization, HCl was added to the solution to establish a pH of 7.5-8.0. This solution was then wrapped in foil and kept at 4°C during the 24 hours of assay. Fresh compound was prepared for each assay. The *in vitro* chemoinvasion analyses for tumor cell invasiveness are performed using the Membrane Invasion Culture System 5 (MICS) as previously described.

The MICS system is a thermally treated plastic manifold system consisting of two matched sets of plates containing fourteen wells, 13 mm in diameter. Interposed between these plates was a polycarbonate filter containing 10 µm pores and coated 10 with a defined matrix composed of human laminin/collagen IV/gelatin that formed a 35 µm thick barrier between the top and bottom sections of the wells in MICS. Prior to placing this barrier in place, the lower wells were filled with serum-free RPMI culture medium made 50% in conditioned medium obtained from 2-day-old cultures of human fibroblasts which had been cleared of cells and cellular debris by either 15 centrifugation, or filtering through a 0.45 µm sterile filter. The barrier was then placed on the lower wells and fresh serum-free medium added to the upper wells after assembly of the system. Fifty thousand tumor cells were then added to the wells either in the presence of the compound, or the DMSO vehicle (the controls). Of the 12 assay wells on each manifold, 3 randomized wells acted as controls, 3 wells 20 received 1 µg/ml of compound, 3 received 10 µg/ml and 3 received 25 µg/ml. After 24 hours, cells and media were removed from the lower wells and replaced by phosphate buffered saline plus 2 mM EDTA. This solution was used to remove all cells from the lower well, and this wash was added to the recovered cells plus medium from the same well. These samples were then collected onto a polylysine containing 25 polycarbonate membrane containing 3 µm pores using a Dot-Blot manifold system. These collection filters were then fixed in methanol, and stained using Wright's stain (Leukostat staining kit) *in situ*. The filters were then mounted on microscope slides with microscope immersion oil (which reduced the light refraction from the pores) and 5 microscopic fields were counted to calculate the number of cells which invaded 30 through the membrane over the 24 hour period. These numbers were then statistically analyzed as discussed below.

TABLE V

COMPOUND NO.	INHIBITORY ACTIVITY OF MATRIX METALLOPROTEINASES (MMPs) ^j					HUMAN CELL LINE EVALUATED AND PERCENT INHIBITION				
	MMP-1	MMP-2	MMP-3	MMP-7	MMP-9	MMP-14	Breast MDA-MB-231	Melanoma C8161	Multiple Myeloma RPMI-8226	Prostate PC-3 ML
1	267	>700	258	815	43	86	7 ^b 20 ^b 3 ^b	5 20 35 ^b	20 10 15	2 47 ^b 60 ^b
2	479	80	348	>1000	117	119	20 15 30 ^b	40 ^b 49 ^b 56 ^b	21 ^b 21 ^b 29 ^b	13 ^b 24 ^b 24 ^b
3	>1000	>1000	304	>1000	136	216	+8 +2 12	20 ^b 25 ^b 27 ^b	+8 +10 ^b +11	20 8 15
4	409	421	219	278	36	61	5 6 2	30 ^b 22 ^b 15 ^b	+7 +23 ^b +22	27 ^b 3 +1 25 ^b
5	647	86	237	441	194	447	6 +4 46 ^b	15 27 ^b 35 ^b	+5 +5 +14	10 +8 3
6	394	135	241	>1000	83	56	+5 +17 ^b 27 ^b	18 ^b 33 ^b 32 ^b	+6 +25 ^b +5	36 44 ^b 35 46 ^b
7	R (5)	>1000	300	335	64	>1000	+18 +41 ^b +27	7 14 33 ^b	12 ^b 10 5	21 26 41 ^b
8	>1000	599	232	388	>1000	>1000	+10 +5 +13	13 ^b 41 ^b 40 ^b	11 3 +2	0 0 +4 0
9	372	337	154	507	28	29				

COMPOUND NO.	INHIBITORY ACTIVITY OF MATRIX METALLOPROTEINASES (MMPs) ^j					HUMAN CELL LINE EVALUATED AND PERCENT INHIBITION				
	MMP-1	MMP-2	MMP-3	MMP-7	MMP-9	MMP-14	Breast MDA-MB-231	Melanoma C8161	Multiple Myeloma RPMI-8226	Prostate PC-3 ML
10	499	484	96	>1000	41	53	9 27 ^b 37 ^b	21 22 11	3 5 29 ^b	40 ^a 50 ^b 65 ^b
11	427	>1000	270	>1000	96	46	+5 0 8	7 28 ^b 30 ^b		9 16 5
12	>1000		390 ^c	503	270	>1000		16 46 ^b 41 ^b		+3 0 36 ^b
13	126	48	206	>1000	64	14	4 0 +9	6 12	14 +11 +13 +12	
14	279	R	>1000	R	R	386				
15	>1000	234	738	716	402	768				
16	452	215	>1000	569	215	715				
17	122	>1000	350		110	98				
18	123	233	335		131	102				

COMPOUND NO	INHIBITORY ACTIVITY OF MATRIX METALLOPROTEINASES (MMPs) ^a					HUMAN CELL LINE EVALUATED AND PERCENT INHIBITION				
	MMP-1	MMP-2	MMP-3	MMP-7	MMP-9	MMP-14	Breast MDA-MB-231	Melanoma C8161	Multiple Myeloma RPMI-8226	Prostate PC-3 ML
19	270	925	394		98	90				
20	429	511	281		162	266				
21	293	>1000	>1000		79	115				
22	396	254	462		341	459				
23	>1000	306	>1000		>1000	>1000				
24	405	724	191		127	160				
25	974	397	303		148	185				
26	>1000	413	>1000		384	586				
27	498	963	149		101	92	15 35 72 ^b	0 +1 73 24 ^b	6 42 ^b 17 ^b 35 ^b	70
28	>1000	>1000	231		125	49	+24+36 ^b 54 ^b	+20 34 ^b 40 ^b	4 +10 34 ^b	7 +11 ^b 80

COMPOUND NO.	INHIBITORY ACTIVITY OF MATRIX METALLOPROTEINASES ¹ (MMPs) ¹					HUMAN CELL LINE EVALUATED AND PERCENT INHIBITION					
	MMP-1	MMP-2	MMP-3	MMP-7	MMP-9	MMP-14	Breast MDA-MB-231	Melanoma C8161	Multiple Myeloma RPMI-8226	+3	Prostate PC-3 ML
29	895	983	213				+7 +15 45 ^b +25 ^b +53 ^b	19	5 +3 62 ^b	35 ^b 28 ^b	61
30	576	>1000	938				+41 ^b 7 59 ^b +13 +25 ^b 17	12 +20 61 ^b	13 12 8 28 ^b 50 ^b	35 ^b 28 ^b	58
31	621	258	>1000				>1000		1 29 ^b 13 35 ^b	8 8	69
32	601	236	244				311	593			
33	295	263	205				142	94			
34	242	242	136				308	197			
35	947	249	218				397	108			
36	539	>1000	283				>1000	608			
37	279	>1000	274				445	41	+6 1 46 ^b	+1 20 69 ^b	10 5 46 ^b
										11 26	36

COMPOUND NO.	INHIBITORY ACTIVITY OF MATRIX METALLOPROTEINASES (MMPs) ^a					HUMAN CELL LINE EVALUATED AND PERCENT INHIBITION					
	MMP-1	MMP-2	MMP-3	MMP-7	MMP-9	MMP-14	Breast MDA-MB-231	Melanoma C8161	Multiple Myeloma RPMI-8226	Prostate PC-3 ML	
38	230	175	95		215	69	+7 37 ^b 70 ^b	3 1 66 ^b	33 ^b 13 +11	37 ^b 41 ^b 41	
39	860	186	682		114	83	18 ^b 29 ^b 76 ^b	10 11 24	5 10 46 ^b	44 ^b 67 ^b 69 ^b	
40	560	362	91		R	29	9 15 20 ^b	+1 +5	+4	16 11 9	
41	478	889	54		250	403	+8 3 2	2 8 6	7 +6	14 +4 4	
42	R	606	1557		405	428					
43	600	2482	174		319						
44	1438	1468	224		591						
45	384	2446	194		6626						
46	194	3803	42		59	90	20 ^b +5 +1	21 7 10	7 +11	12 20 16 27 ^b	

COMPOUND NO.	INHIBITORY ACTIVITY OF MATRIX METALLOPROTEINASES (MMPs) ¹					HUMAN CELL LINE EVALUATED AND PERCENT INHIBITION				
	MMP-1	MMP-2	MMP-3	MMP-7	MMP-9	MMP-14	Breast MDA-MB-231	Melanoma C8161	Multiple Myeloma RPMI-8226	Prostate PC-3 ML
47	318	117	68		88	60	+17 +16 ^b	0	+30 +4	+2
48	12831	1929	506		424	354			28 18 60 ^b	3 3 5
49	372	1572	141		489	87	6 +5 +1	27 ^b 7	30 ^b 11 +2	15 4 +5 6
50	211	2533	48		34	40	7 0 2	5 +2	1 +14 +8	+13 +14 +17 +10
51							+15 ^b +18 25 ^b	+9 +31 ^b +122 ^b	14 +26 ^b +2	+4 +13 4
52	>1000	250	>1000	>1000	32	68	5 12 52 ^b	5 15	30	2 26 ^b 68 ^b 52 ^b 68 ^b 70 ^b
53							9 49 ^b 83 ^b +40 ^b +158 ^b	83 ^b +95 ^b	18 84 ^b 29 ^b 21 45 ^b	

COMPOUND NO.	INHIBITORY ACTIVITY OF MATRIX METALLOPROTEINASES ¹ (μmMps) ¹						CELL LINE EVALUATED AND PERCENT INHIBITION ^A			
	MMP-1	MMP-2	MMP-3	MMP-7	MMP-9	MMP-14	RPMI-8226	ARP-1	ARK	
1	267	>700	258	815	43	86	20 21 ^B	10 29 ^B	+9 +13	1
2	479	80	348	>1000	117	119	+8 1 ^B	+10 ^B +4 ^B	+11 7 ^B	10 10
3	>1000	>1000	304	>1000	136	216	+7 +22	+23 ^B +3	+27 ^B +24 ^B	7 +13
5	647	86	237	41	194	447	6 17	4 37 ^B	36 42 ^B	+16 +13
6	394	135	241	<1000	83	56	+6 12 ^B	+25 ^B 10	+5 5	+11 NE
9	372	337	154	507	28	98	21 ^B	24 ^B	28 ^B	+7 +14
10	499	484	96	>1000	41	53	3	5	29 ^B	+7 NE
13	126	48	206	>1000	64	14	+11	+13	+12	+7 NE
										2 2

For Table V:

^Aconcentrations of 1, 10 and 25 μg/mL were used
^BSignificant, i.e., P<0.05

5

+ = increased invasiveness

NP = not evaluated

R = to be repeated (number times repeated)

¹IC₅₀ in μM unless specified otherwise
²Predicted

Key to Table V:

Compound	Substance
1:	9-nitro-6-deoxy-5-hydroxytetracycline H ₂ SO ₄
2:	9-amino-6-deoxy-5-hydroxytetracycline H ₂ SO ₄
3:	9-Isopropylamino-6-deoxy-5-hydroxytetracycline H ₂ SO ₄
4:	7-Dimethylamino-6-demethyl-6-deoxy-9-nitrotetracycline H ₂ SO ₄
5:	Same as 2
6:	9-Azido-6-deoxy-5-hydroxytetracycline H ₂ SO ₄
7:	9-Amino-7-dimethylamino-6-demethyl-6-deoxytetracycline H ₂ SO ₄
8:	9-Acetamido-7-dimethylamino-6-demethyl-6-deoxytetracycline H ₂ SO ₄
9:	7-dimethylamino-6-demethyl-6-deoxytetracycline-9-diazonium H ₂ SO ₄ HCL
10:	9-Azido-7-demethylamino-6-demethyl-6-deoxytetracycline H ₂ SO ₄
11:	6-Deoxy-5-hydroxytetracycline-9-diazonium H ₂ SO ₄
12:	7-Amino-6-deoxy-5-hydroxytetracycline H ₂ SO ₄
13:	7,9-Dibromo-6-demethyl-6-deoxytetracycline H ₂ SO ₄
14:	7-Amino-6-deoxy-5-hydroxy-9-nitrotetracycline H ₂ SO ₄
15:	7-Dimethylamino-6-deoxy-5-hydroxy-tetracycline H ₂ SO ₄
16:	9-Acetamido-6-deoxy-5-hydroxytetracycline H ₂ SO ₄
17:	7-Di-n-Butylamino-6-deoxy-5-hydroxytetracycline H ₂ SO ₄
18:	7-Di-n-Hexylamino-6-deoxy-5-hydroxytetracycline H ₂ SO ₄
19:	7-Di-(3,3-dimethylbutyl)amino-6-deoxy-5-hydroxytetracycline H ₂ SO ₄
20:	7-Azido-6-deoxy-5-hydroxy-tetracycline H ₂ SO ₄
21:	6-Deoxy-5-hydroxytetracycline-7-diazonium H ₂ SO ₄
22:	7-Acetamido-9-nitro-6-deoxy-5-hydroxytetracycline H ₂ SO ₄
23:	7-Acetamido-6-deoxy-5-hydroxytetracycline H ₂ SO ₄
24:	7-Di-n-propylamino-6-deoxy-5-hydroxytetracycline H ₂ SO ₄
25:	7-Isobutylamino-6-deoxy-5-hydroxytetracycline H ₂ SO ₄
26:	7-Acetamido-9-amino-6-deoxy-5-hydroxytetracycline H ₂ SO ₄
27:	7-Isobutylmethylamino-6-deoxy-5-hydroxytetracycline H ₂ SO ₄

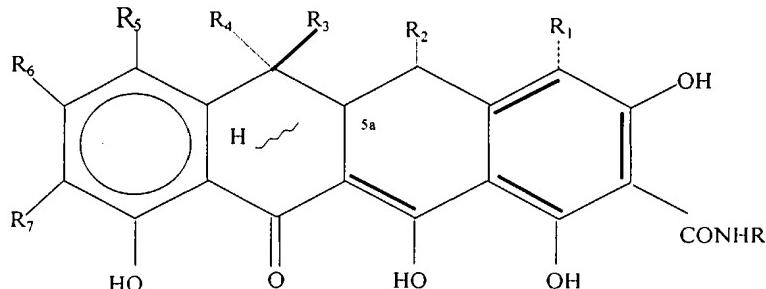
- 28: 6-Deoxy-5-acetoxy-tetracycline H₂SO₄
- 29: 7-Acetylisobutylamino-6-deoxy-5-hydroxytetracycline H₂SO₄
- 30: 7-Acetamido-6-deoxy-5-hydroxytetracycline-9-diazonium H₂SO₄
- 31: 7-Dimethylamino-6-deoxy-5-hydroxytetracycline-9-diazonium H₂SO₄
- 32: 7-Cyclobutylamino-6-deoxy-5-hydroxytetracycline H₂SO₄
- 33: 7-Cyclobutylmethylamino-6-deoxy-5-hydroxytetracycline H₂SO₄
- 34: 4-Dedimethylamino-6-deoxy-5-hydroxytetracycline
- 35: 4-Dedimethylamino-6-deoxy-5-hydroxy-9-nitrotetracycline
- 36: 9-animo-4-dedimethylamino-6-deoxy-5-hydroxytetracycline sulfate
- 37: 4-Dedimethylamino-6-deoxy-5-hydroxytetracycline-9-diazonium sulfate
- 38: 7-nitro-9-tertbutyl-6-deoxy-5-hydroxytetracycline H₂SO₄
- 39: 9-tertbutyl-6-deoxy-5-hydroxytetracycline H₂SO₄
- 40: 7-Nitro-9-tertbutyl-4-dedimethylamino-6-deoxy-5-hydroxytetracycline H₂SO₄
- 41: 4-Dedimethylamino-7-dimethylamino-6-demethyl-6-deoxytetracycline H₂SO₄
- 42: 7-Acetylamino-9-azido-6-deoxy-5-hydroxy-tetracycline
- 43: 9-tertbutyl-6-deoxy-5-hydroxytetracycline-7-diazonium H₂SO₄
- 44: 7-Amino-9-tertbutyl-6-deoxy-5-hydroxytetracycline H₂SO₄
- 45: 7-Azido-9-tertbutyl-6-deoxy-5-hydroxytetracycline H₂SO₄
- 46: 6-deoxy-5-hydroxytetracycline-4-Methiodide
- 47: 7-(1,2-benzylcarboxyhydrazine)-6-deoxy-5-hydroxy-tetracycline HCl
- 48: 4-Dedimethylamino-7-amino-6-deoxy-5-hydroxyltetracycline H₂SO₄
- 49: 7-Amino-9-tertbutyl-4-dedimethyl-6-deoxy-5-hydroxytetracycline-H₂SO₄
- 50: 4-Dedimethylamino-9-tertbutyl-6-deoxy-5-hydroxytetracycline-7-diazonium H₂SO₄
- 51: 9-(4-fluorophenyl)-6-deoxy-5-hydroxytetracycline
- 52: 4-Epi-7-Chlorotetracycline hydrochloride
- 53: 6-Demethyl-6-deoxytetracycline HCl

[000104] While the present invention has been described with respect to particular embodiment thereof, it is apparent that numerous other forms and modifications of the invention will be obvious to those skilled in the art. The appended claims and this invention generally should be construed to cover all such obvious forms and 5 modifications, which are within the true spirit and scope of the present invention.

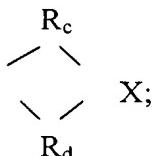
What is claimed is:

1. A treatment for inhibiting microbial or tumor growth which comprises adding to a subject in need of said treatment an effective amount of a tetracycline composition or salt thereof, comprising a general formula:

5



wherein R is selected from $\text{CH}_2\text{N}(\text{R}_a\text{R}_b)$ or CH_2N



10

R_aR_b are C_1 to C_6 alkyl;

R_c and R_d are $(\text{CH}_2)_n \text{CHR}_e$, n is 0 or 1 and R_e is H, C_1 to C_6 alkyl, or NH_2 , and X is NH, S, or CH_2 ;

R_1 is H or OH;

15 R_2 is H, OH, =O, or OCOR_8 , where R_8 is C_1 to C_6 alkyl, or alternatively, R_2 is $\text{N}(\text{R}_9)_2$, where R_9 is hydrogen or C_1 to C_6 lower alkyl, or R_9 CO, with the proviso that when R_2 is keto or $\text{N}(\text{R}_9)_2$, then R_3 and R_4 are H or CH_3 , and R_3 and R_4 are not both CH_3 or H;

5a hydrogen is α or β ;

20 R_3 and R_4 are H, CH_3 or F, with the proviso that when R_3 is CH_3 , then R_4 is H or F, and when R_4 is CH_3 , then R_3 is H or F and R_4 and R_5 are not both F;

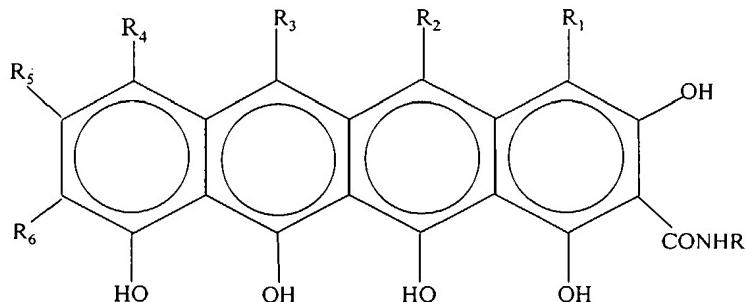
R_5 and R_7 are halogen, H, NO_2 , N_3 , N_2^+ , $\text{C}_2\text{H}_5\text{OC}(\text{S})\text{S}-$, CN, $\text{NR}_{10}\text{R}_{11}$, where R_{10} and R_{11} are H, C_1 to C_{10} alkyl, and $\text{R}_{12}(\text{CH}_2)_n\text{CO}-$, where n is 0 to 5 and R_9 is H, NH_2 , mono or disubstituted amino selected from straight, branched or cyclized C_1 to C_{10}

25 alkyl groups, with the proviso that R_{10} and R_{11} are not both $\text{R}_{12}(\text{CH}_2)_n\text{CO}-$; R_7 , alternatively, is tertiary butyl; and

R_6 is halogen, acetylene, $\text{R}_{13}\text{-C}_6\text{H}_5$, where R_{13} is NO_2 , halogen, acetylamino, amino, phenyl, alkyl, or alkoxy.

2. A treatment for inhibiting microbial or tumor growth which comprises adding to a subject in need of said treatment an effective amount of a tetracycline composition or salt thereof, comprising a general formula:

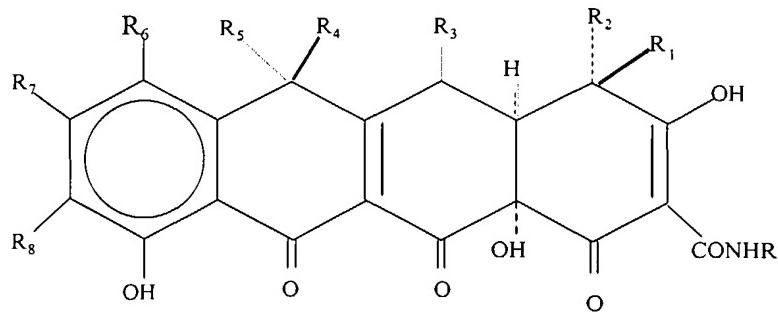
5



wherein R is selected from $\text{CH}_2\text{N}(\text{R}_a\text{R}_b)$ or $\text{CH}_2\text{N} \begin{array}{c} \diagup \\ \diagdown \end{array} \text{X};$
 $\begin{array}{c} \diagup \\ \diagdown \end{array}$ R_c
 R_d

- 10 where R_aR_b are C_1 to C_6 alkyl;
 R_c and R_d are $(\text{CH}_2)_n \text{CHR}_e$, n is 0 or 1 and R_e is H, C_1 to C_6 alkyl, or NH_2 , and X is NH, S, or CH_2 ;
 R_1 is H or OH;
 R_2 is H or OH;
15 R_3 is H or CH_3 ;
 R_4 and R_6 are halogen, H, NO_2 , N_3 , N_2^+ , $\text{C}_2\text{H}_5\text{OC(S)S-}$, CN, NR_7R_8 , where R_7 and R_8 are H, C_1 to C_{10} alkyl, and $\text{R}_9(\text{CH}_2)_n\text{CO-}$, where n is 0 to 5 and R_9 is H, NH_2 , mono or disubstituted amino selected from straight, branched or cyclic $\text{C}_1\text{-C}_{10}$ alkyl, with the proviso that R_7 and R_8 are not both $\text{R}_9(\text{CH}_2)_n\text{CO-}$; and
20 R_6 is H or halogen, acetylene, $\text{R}_{10}\text{-C}_6\text{H}_5$, where R_{10} is NO_2 , halogen, acetylamino, amino, phenyl, alkyl, or alkoxy.

3. A treatment for inhibiting microbial or tumor growth which comprises adding to a subject in need of said treatment an effective amount of a tetracycline composition or salt thereof, comprising a general formula:



wherein R is selected from $\text{CH}_2\text{N}(\text{R}_a\text{R}_b)$ or $\text{CH}_2\text{N} \begin{array}{c} \diagup \\ \diagdown \end{array} \text{X};$
 $\begin{array}{c} \diagup \\ \diagdown \end{array}$ R_c
 R_d

5 where R_aR_b are $\text{C}_1\text{-}\text{C}_6$ alkyl;

R_c and R_d are $(\text{CH}_2)_n \text{CHR}_e$, n is 0 or 1 and R_e is H, C_1 to C_6 alkyl, or NH_2 , and X is NH, S, or CH_2 ;

R_1 and R_2 are H or $\text{N}(\text{R}_9)_2$, where R_9 is H or C_1 to C_6 alkyl with the proviso that R_1 and R_2 are not both $\text{N}(\text{R}_9)_2$, R_1 and R_2 are also $\text{N}(\text{R}_{10})_3$ I, where R_{10} is C_1 to C_6 alkyl,

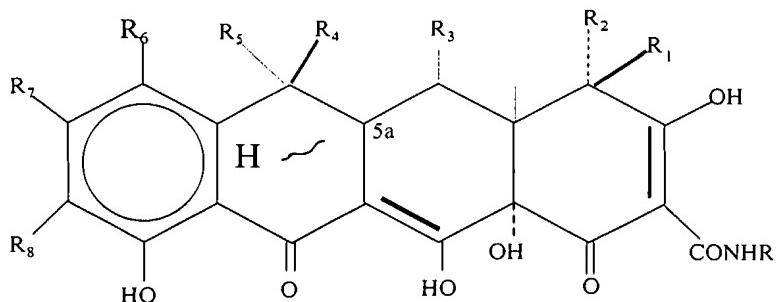
10 with the proviso that R_1 and R_1 are not both $\text{N}(\text{R}_{10})_3$ I;

R_3 is H;

R_4 and R_5 are H, CH_3 or F, with the proviso that when R_4 is CH_3 , then R_5 is H or F, and when R_5 is CH_3 , then R_4 is H or F, and R_4 and R_5 are not both F;

15 R_6 and R_8 are halogen, H, NO_2 , N_3 , N_2^+ , $\text{C}_2\text{H}_5\text{OC(S)S-}$, CN, $\text{NR}_{11}\text{R}_{12}$, where R_{11} and R_{12} are H, C_1 to C_{10} alkyl, and $\text{R}_{13}(\text{CH}_2)_n\text{CO-}$, where n is 0 to 5 and R_{13} is H, NH_2 , mono or disubstituted amino selected from straight, branched or cyclized $\text{C}_1\text{-}\text{C}_{10}$ alkyl groups, with the proviso that R_{10} and R_{11} are not both $\text{R}_{12}(\text{CH}_2)_n\text{CO-}$; and
 R_8 can also be tertiary butyl and R_7 is H or halogen.

20 4. A treatment for inhibiting microbial or tumor growth which comprises adding to a subject in need of said treatment an effective amount of a tetracycline composition or salt thereof, comprising a general formula:



wherein R is selected from $\text{CH}_2\text{N}(\text{R}_a\text{R}_b)$ or $\text{CH}_2\text{N} \begin{array}{c} \text{R}_c \\ \diagup \\ \diagdown \\ \text{R}_d \end{array} \text{X}$;

5 R_aR_b are $\text{C}_1\text{-C}_6$;

R_c and R_d are $(\text{CH}_2)_n\text{CHR}_e$, n is 0 or 1, R_e is H, alkyl (C_1 to C_6), NH_2 , and X is NH, S, or CH_2 ;

10 R_1 and R_2 are H or $\text{N}(\text{R}_9)_2$ where R_9 is hydrogen or lower alkyl (C_1 to C_6), with the proviso that R_1 and R_2 can not both be $\text{N}(\text{R}_9)_2$.

15 R_3 is H, OH, =O, OCOR_{10} , where R_{10} is C_1 to C_6 , or alternatively, R_3 is $\text{N}(\text{R}_9)_2$, where R_9 is hydrogen or C_1 to C_6 , with the proviso that when R_{23} is =O or $\text{N}(\text{R}_9)_2$, then R_4 and R_5 are H or CH_3 and R_4 and R_5 are not both CH_3 or H;

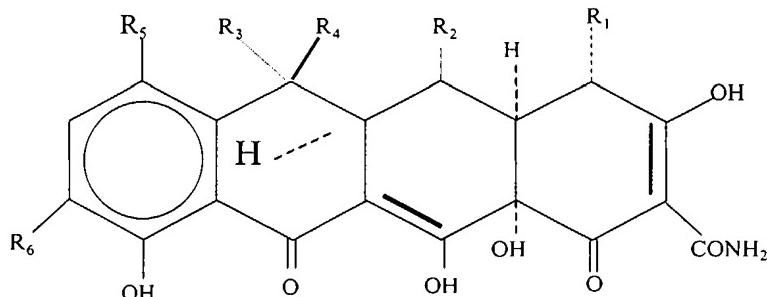
5a hydrogen is α or β ;

R_4 and R_5 are H, CH_3 or F, with the proviso that when R_4 is CH_3 , then R_5 is H or F, and when R_5 is CH_3 then R_4 is H or F and R_4 and R_5 are not both F;

20 R_6 and R_8 are halogen, H, NO_2 , N_3 , N_2^+ , $\text{C}_2\text{H}_5\text{OC}(\text{S})\text{S}-$, CN, $\text{NR}_{10}\text{R}_{11}$, where R_{10} and R_{11} are H, alkyl (C_1 to C_{10}), and R_{12} $(\text{CH}_2)_n\text{CO}-$, where n is 0 to 5 and R_{12} is H, NH_2 , mono or disubstituted amino selected from straight, branched or cyclized C_1 to C_{10} groups, with the proviso that R_{10} and R_{11} are both R_{12} $(\text{CH}_2)_n\text{CO}-$, or, alternatively, R_8 is tertiary butyl;

25 R_7 is H or halogen, acetylene, $\text{R}_{12}\text{-C}_6\text{H}_5$, where R_{12} is NO_2 , halogen, acetylamino, amino, phenyl, alkyl, or alkoxy.

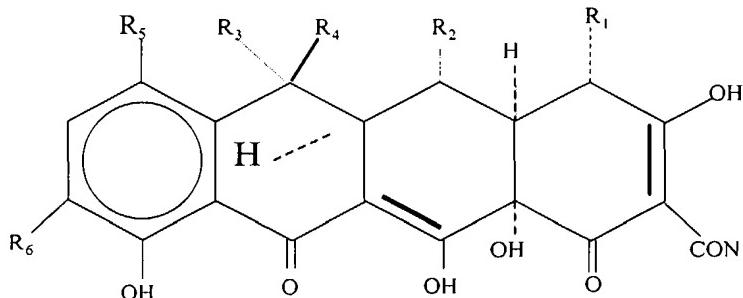
5. A treatment for inhibiting microbial or tumor growth which comprises adding to a subject in need of said treatment an effective amount of a tetracycline composition or salt thereof, comprising a general formula:



5

wherein R₁ is H or N(CH₃)₂; R₂ is H, CH₃ or OCOCH₃; R₃ is H or CH₃; R₄ is H; R₅ is H, N(CH₃)₂, NH₂, N(C₄H₉)₂, N(C₆H₁₃)₂, N(3,3 - dimethylbutyl)₂, N₃, N₂⁺, NO₂, NHCOCH₃, NH(n-propyl)₂, NH-isobutyl, NH-isobutylmethyl, NH(cyclobutyl), 10 NH(cyclobutyl methyl); and R₆ is H, NO₂, NH₂, (CH₃)₂ CHNH, N₃, NHCOCH₃, N₂⁺, N₃ or (CH₃)₃C.

6. A treatment for inhibiting microbial or tumor growth which comprises adding to a subject in need of said treatment an effective amount of a tetracycline composition or salt thereof, comprising a general formula:



wherein R₁ is H or N(CH₃)₂; R₂ is H, OH or OCOCH₃; R₃ is H or CH₃; R₄ is H; R₅ is H, N(CH₃)₂; N(C₄H₉)₂, N(C₆H₁₃)₂, N(3,3-dimethylbutyl)₂; N₃, N₂⁺, NO₂, NHCOCH₃, 20 NH(n-propyl)₂, NH-isobutyl, NH-isobutylmethyl, N(CH₃CO)(isobutyl), NH(cyclobutyl), NH(cyclobutyl methyl), or NH₂; and R₆ is H, NO₂, NH₂, (CH₃)₂ CHNH, N₃, NHCOCH₃, N₂⁺, or (CH₃)₃C.